Activated Macrophages in Repair of Skeletal Muscle Injuries

BY

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THESIS

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βIG-H3	TGFβ-inducible gene-H3
CFSE	Carboxyfluorescein succinimidyl ester
CM	Conditioned medium
DAMP	Damage-associated molecular pattern
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HGF	Hepatocyte growth factor
IFNγ	Interferon γ
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MMP	Matrix metalloprotease
Mp	Macrophage
M1	Cultured Mp activated with IFNγ and TNFα
M2a	Cultured Mp activated with IL-4 or IL-13
M2c	Cultured Mp activated with IL-10
PAI-1	Plasminogen activator inhibitor-1
RICE	Rest-ice-compression-elevation

LIST OF ABBREVIATIONS (continued)

TGFβ	Transforming growth factor β
TNFα	Tumor necrosis factor α
uPA	Urokinase Type Plasminogen Activator

SUMMARY

Macrophages (Mp) are essential for repair and regeneration in numerous tissues, including skin, skeletal and cardiac muscle, and liver (1-5). However, chronic inflammation and persistent Mp accumulation are often associated with tissue destruction and fibrosis (6-8). These contrasting roles of Mp may be due to the ability of Mp to assume a large spectrum of functional phenotypes upon exposure to different activating stimuli (9). Two of the best-characterized *in vitro* phenotypes of Mp are a pro-inflammatory "M1" phenotype produced by exposure to interferon (IFN)γ and tumor necrosis factor (TNF)α, and an anti-inflammatory "M2a" phenotype produced by exposure to interleukin (IL)-4 or IL-13 (10). Because cultured M2a Mp produce numerous factors that are known to be important in tissue repair, M2a Mp are frequently termed "wound healing" Mp (9), and are widely assumed to participate in tissue repair processes (6,9,11,12), though direct evidence for M2a Mp *in vivo* is lacking. The guiding hypothesis of this project was that Mp after traumatic skeletal muscle injury exhibit a transition from an M1 to an M2a phenotype, and that modulation of Mp phenotype can be used to promote regeneration and reduce fibrosis in injured muscle.

Data presented here demonstrate that, in a mouse model of skeletal muscle laceration injury, formation of a persistent collagenous scar is accompanied by prolonged Mp accumulation lasting at least 21 days. However, Mp did not exhibit either of the *in vitro*-defined M1 or M2a phenotypes. Gene expression profiling of muscle Mp after injury revealed expression of markers of

both M1 and M2a activation at early time points after injury, followed by a general deactivation during later stages of repair. In particular, Mp expression of IL-10 mRNA and protein was markedly increased at 3 days post-injury, and we speculate that this powerful anti-inflammatory cytokine may contribute to subsequent deactivation of Mp. Flow cytometry analysis revealed that the mixed M1/M2a gene expression profile at 3 days post-injury was due to a hybrid phenotype of individual Mp, rather than simultaneous presence of both M1 and M2a populations.

Though an M1 phenotype was not observed in endogenous Mp during muscle repair, treatment of lacerated skeletal muscle with exogenous M1 Mp dose-dependently reduced fibrosis after injury. Injection of 2 million M1 activated Mp resulted in a significant decrease in collagen accumulation at 14 days post-injury, injection of 1 million Mp produced a non-significant trend for decreased collagen, and injection of 0.5 million Mp did not alter collagen accumulation relative to saline-injected controls. Additionally, M1 activation was necessary for this therapy to be effective, because injection of 2 million non-activated Mp had no significant effect on collagen accumulation versus saline control. However, neither M1 nor non-activated Mp significantly altered myofiber regeneration. Future studies may investigate whether combination of Mp cell therapy with other treatments, such as growth factors or stem cells, can enhance muscle regeneration in addition to preventing fibrosis.

We also investigated whether production of urokinase-type plasminogen activator (uPA) by M1 Mp mediates the effects of these cells on cultured

myoblasts and fibroblasts. uPA is known to promote skeletal muscle regeneration and may also be anti-fibrotic (13-15). We found that conditioned medium (CM) from M1 Mp increased proliferation of cultured myoblasts and decreased collagen gene expression in cultured fibroblasts. While previous studies have similarly demonstrated that myoblast proliferation is increased by soluble factors produced by Mp (16,17), the finding that a soluble factor (rather than cell-cell contacts in coculture) also reduces fibroblast collagen production is novel. However, this soluble factor does not appear to be uPA. Despite previous reports of increased uPA in Mp that have been activated to an M1 phenotype (14,18), we detected no active uPA in CM from M1 activated Mp. Additionally, myoblast proliferation in the presence of M1 Mp-CM was not altered by addition of the uPA inhibitor plasminogen activator inhibitor (PAI)-1. These results demonstrate that, despite the ability of Mp-derived uPA to promote muscle regeneration and reduce fibrosis *in vivo* (13), uPA is not necessary for the alterations of cultured myoblast and fibroblast activity induced by M1 Mp.

In conclusion, data presented in this dissertation demonstrate that, during repair of lacerated skeletal muscle, Mp exhibit temporally regulated phenotypes that cannot be classified as strictly M1 or M2a. Muscle Mp transition from an early hybrid phenotype expressing both M1- and M2a-associated markers to an apparently deactivated phenotype as repair progresses. Additionally, cell therapy with M1 Mp reduces collagen accumulation in lacerated muscle. Despite the known importance of uPA in muscle regeneration, uPA does not appear to mediate the effects of M1 Mp on activity of cultured myoblasts and fibroblasts. An

improved understanding of the reciprocal regulation of Mp phenotype and the tissue repair environment may assist in development of novel therapies to promote healing.

CHAPTER 1

INTRODUCTION

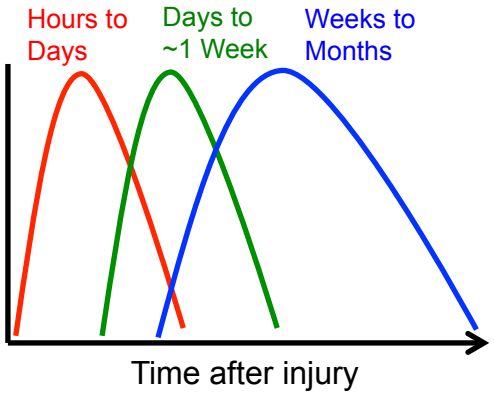
A. SKELETAL MUSCLE INJURY AND REPAIR

Skeletal muscle injuries are some of the most common injuries encountered in sports medicine (19), and can also result from accidents or military combat. Muscle repair after traumatic injury or exercise-induced tears is prolonged and incomplete. Return to pre-injury activity levels often takes months, while injured muscle tissue is replaced by fibrotic scar tissue, which persists indefinitely (20). Muscle force production is decreased due to the non-contractile nature of the scar, and the scar site is highly susceptible to re-injury (20). Current treatments of muscle injury, such as RICE (rest-ice-compression-elevation), focus on restoring hemostasis and preserving tissues surrounding the site of injury; methods for improving regeneration or reducing scarring are largely lacking. Long-term scarring after muscle injury remains a common problem despite current rehabilitation protocols (21). In order to develop improved treatments for skeletal muscle injuries, a better understanding of the cellular and molecular mechanisms underlying both muscle regeneration and fibrosis is necessary.

1. Phases of skeletal muscle repair

Repair of skeletal muscle and other tissues occurs in overlapping phases of inflammation, proliferation, and remodeling [Figure 1] (19,22). Within hours

after injury, inflammatory cells invade the injured muscle, where they clear necrotic tissue and release soluble mediators that promote regeneration. Meanwhile, muscle satellite cells are activated, proliferate, and fuse to form new myofibers, which enlarge and mature to restore tissue structure and function. Simultaneously, however, production of extracellular matrix (ECM) proteins by fibroblasts can result in formation of a fibrous scar at the site of injury. This scar fills the defect created by trauma or tear, and joins the remaining muscle stumps on either side of the injured area. Thus the scar allows partial restoration of muscle function but may also impede formation of regenerating myofibers at the injured site (19). The balance between new myofiber formation and ECM deposition by fibroblasts is thought to determine whether the healing outcome is primarily regenerative or fibrotic (23).



- Inflammatory Phase: Hemostasis; neutrophil and macrophage accumulation; phagocytosis of necrotic tissue
- Proliferative Phase: Activation, proliferation, and fusion of myoblasts; fibroblast proliferation; angiogenesis; matrix deposition
- Remodeling Phase: Matrix deposition and remodeling; vascular remodeling; hypertrophy of regenerating myofibers

Figure 1. Phases of muscle healing

2. Rodent models of muscle regeneration and fibrosis

Experimental rodent models of muscle injury demonstrate that skeletal muscle has an enormous potential for regeneration, even if this potential is not fully realized after human injuries. Injection of chemical toxins produces severe muscle injury, with an almost complete loss of myofiber integrity throughout the entire muscle within the first few days after injection (24,25). Soon, regenerating centrally nucleated muscle fibers begin to repopulate the muscle, and regeneration and restoration of muscle function are nearly complete by about 20 days post-injury (24,25). Additionally regeneration in this toxin-induced injury model progresses with minimal fibrosis (26). In contrast, rodent models of muscle injury employing laceration, contusion, or strain more closely mimic the human situation and heal by a combination of regeneration and fibrosis (23,27-30). In these models, muscle force production at four weeks post-injury is only restored to about half of uninjured muscle strength (28,29), and collagenous scars persist for at least 56 days (27). It remains to be determined why toxin-induced experimental injuries regenerate completely while traumatic injuries in rodents and humans heal by scar formation and only partial regeneration.

3. Therapies to improve muscle healing

Common treatments for muscle injury are very conservative, and are generally limited to RICE for restoration of hemostasis and pain management, and early remobilization to promote functional recovery (19,20,31,32). Yet even after treatment and rehabilitation, persistent scar formation remains problematic

(21). A number of therapies have been shown to improve healing in experimental animal models of traumatic or exercise-induced muscle injury, but most of these therapies have yet to be translated to the clinic. Anti-fibrotic agents may enhance muscle regeneration in animal models; decorin, relaxin, and suramin, all of which have anti-fibrotic effects through blockade of transforming growth factor (TGF)β activity, increase myofiber regeneration and force recovery after traumatic injury (23,33,34). Combination of anti-fibrotic agents with growth factor therapy may further improve healing (29). Additionally, injection of Mp-conditioned medium into excisionally wounded rat muscle enhances regeneration and reduces fibrosis (17). Though the specific substances released by Mp to promote healing remain to be identified, secreted growth factors or proteases may be responsible. Extracellular proteases such as matrix metalloprotease (MMP)-1 and the urokinase-type plasminogen activator (uPA) both appear to reduce fibrosis and enhance regeneration in mouse models of muscle injury (13,14,35,36). An improved understanding of the mechanisms of muscle regeneration and fibrosis may help to develop novel therapies and translate these treatments into clinical practice.

4. Macrophages in muscle regeneration

Though chronic inflammation is commonly associated with tissue damage and fibrosis, acute inflammation after injury is necessary for efficient healing in numerous tissues, including skeletal muscle (2,3,5,14,37,38). Within hours after injury, circulating neutrophils and monocytes invade the injured muscle (39,40).

Neutrophil accumulation peaks within the first few days after injury (24,41), and these cells may cause collateral tissue damage through production of reactive oxygen species (40). Neutrophils also release pro-inflammatory cytokines and chemoattractants that promote recruitment of additional leukocytes, including monocyte/macrophages, into the injured muscle (40). Macrophage (Mp) accumulation peaks as neutrophil accumulation is declining, at around 3-7 days post-injury (24,39,41).

Mp are essential for skeletal muscle regeneration. Depletion of Mp after skeletal muscle injury impedes clearance of necrotic tissues and reduces the number and diameter of regenerating myofibers (3,14,39). In addition to their classical function of phagocytosing dead tissues, Mp can actively promote skeletal muscle regeneration by modulating myoblast activity. For instance, cell-cell contacts between Mp and myoblasts enhance survival of both cell types in culture (16). Mp also produce soluble factors that enhance myoblast proliferation (16,17). Additionally, differently activated Mp can promote proliferation, differentiation, and/or fusion of myoblasts (39). However, the specific mechanisms by which Mp promote myoblast activity and muscle regeneration are poorly understood. For example, the soluble factors released by Mp to promote myoblast proliferation remain unidentified. Additionally, Mp are capable of promoting both regeneration and fibrosis in muscle and other tissues (3,12,14,39,42-44); the mechanisms behind these contrasting roles Mp are not fully understood, but may be related to the remarkable functional and phenotypic plasticity displayed by Mp upon exposure to different microenvironments (9).

Importantly, while Mp depletion is detrimental to muscle regeneration, the potential for enhancement of regeneration by Mp cell therapy or modulation of Mp activity has not yet been realized.

B. MACROPHAGE PHENOTYPES DURING TISSUE REPAIR

1. Introduction

Across diverse tissues, repair after injury occurs in overlapping phases of inflammation, proliferation, and remodeling. Within hours after injury, circulating leukocytes invade the injury site, where they participate in host defense, phagocytosis of necrotic tissue, and secretion of paracrine factors. Macrophages (Mp) in particular are known to be essential to regeneration, repair, and remodeling in many tissues, including skin, skeletal and cardiac muscle, and liver (1-5). However, chronic inflammation and persistent Mp accumulation are often associated with tissue destruction and fibrosis (6-8).

These seemingly contradictory roles of Mp in tissue injury and repair are likely related to the ability of Mp to assume markedly different phenotypes in response to specific environmental cues. Mp have long been known to exhibit a pro-inflammatory phenotype, now termed “classically activated” or “M1,” in response to bacterial components or IFNγ and TNFα; however, Mp can also assume a variety of “alternatively activated” or “M2” phenotypes in response to stimuli such as IL-4, glucocorticoids, and IgG complexes (9). Because *in vitro* IL-4 treatment of Mp causes an upregulation of TGFβ and arginase, IL-4-activated

Mp have also been termed “wound healing” Mp, and are widely assumed to participate in tissue repair processes (6,9,11,12). However, much of the defining work on alternative Mp activation has been performed *in vitro* using single cytokines or a small number of stimuli in combination, whereas damaged tissue contains myriad factors that could influence Mp behavior in unexpected ways. Indeed, even in *in vitro* studies, combinations of only two cytokines can produce a Mp phenotype distinct from that elicited by either cytokine alone, and Mp remain responsive to new stimuli after their initial activation (45). The actual factors influencing Mp phenotype during *in vivo* tissue injury and repair remain largely undetermined.

This review will address recent advances in our understanding of Mp phenotypes during tissue repair, and will argue that the Mp populations that participate in tissue repair are not analogous to *in vitro* IL-4-activated Mp. Instead, Mp present during tissue repair are not a single homogenous population, but include a temporally regulated spectrum of activation states to orchestrate the various phases of healing. Improved understanding of the regulation of Mp phenotype both *in vivo* and *in vitro* may help to generate novel therapies to enhance tissue repair.

2. Evolving macrophage activation paradigms

Our understanding of Mp biology has improved immensely over the past few decades, and new discoveries continue to redefine paradigms of Mp activation. Until relatively recently, Mp activation was viewed as a matter of on/off

switching: naïve Mp were thought to become activated upon exposure to bacterial components or IFNγ and TNFα, which increase Mp production of inflammatory cytokines and reactive oxygen species and enhance their pathogen-killing capacity. In contrast, the primary effect of anti-inflammatory cytokines on Mp was thought to be deactivation and resolution of inflammation (46). However, in 1992, Stein and colleagues demonstrated that IL-4 is not merely an off signal for Mp immune activation, but also increases expression of the Mp mannose receptor (MMR, also called CD206) and enhances endocytosis of mannosylated ligands (47). Therefore, IL-4-treated Mp were termed "alternatively activated," to contrast with both "classical" activation by IFNγ/TNFα and the general deactivation that was previously thought to occur with IL-4 treatment.

Subsequently, additional stimuli that were previously considered to be Mp deactivators were also shown to produce distinct Mp activation phenotypes *in vitro*. The term "alternative" activation has expanded to include these new phenotypes, now frequently referred to as M2a/b/c, while "classical" activation is referred to as M1 (10). The M2a phenotype is produced *in vitro* by exposure to IL-4 or IL-13, which act through the common receptor IL-4Rα to increase expression of CD206, arginase, and TGFβ (48-51). The M2b phenotype is produced by exposure to a combination of IgG immune complexes and lipopolysaccharide (LPS), which increases production of IL-10 and decreases production of IL-12, imparting potent anti-inflammatory properties (45,52). *In vitro* exposure to IL-10 or glucocorticoids produces the M2c phenotype, which is

similarly characterized by high IL-10 and low IL12 production (45), as well as increased cell-surface expression of the scavenger receptor CD163 (53,54). Mp phenotype can also be altered by phagocytosis of apoptotic or necrotic cells (55-57). Together, these studies have demonstrated that, far from being merely turned “on” or “off” by pro-inflammatory versus anti-inflammatory cytokines, Mp can adopt a variety of different phenotypes in response to different stimuli.

While discrete M1/2a/b/c phenotypes can certainly be produced *in vitro*, Mp phenotype is by no means restricted to these four categories. Indeed, the actual phenotypes acquired by Mp, even *in vitro*, depend upon their stage of differentiation, the total biochemical milieu, and the dose and duration of the activating stimuli (45,58,59). Additionally, Mp remain responsive to further stimuli after their initial activation (45,60). Thus, available evidence indicates that M1 and M2a/b/c are not discrete Mp differentiation states, but instead represent convenient but limited and arbitrary *in vitro*-defined reference points on a time-dependent and multi-dimensional continuum of possible Mp phenotypes and functions.

3. Evidence for M2a macrophages as tissue repair macrophages

Mosser and Edwards have advocated a functional spectrum or “color wheel” as a model for Mp activation, rather than categorization by phenotypic markers (9). In this model, the “primary colors” represent three of the major functions of Mp: host defense, immune regulation, and wound healing. Far from being restricted to a single function or “color,” Mp may assume multiple functions

simultaneously in the same tissue, and this is represented by primary colors combining to form secondary colors of all shades. In describing three primary functions of Mp, Mosser and Edwards roughly equate “host defense” Mp with the IFNγ/TNFα-activated M1 phenotype, “regulatory” Mp with the IL-10-activated M2c phenotype, and “wound healing” Mp with the IL-4-activated M2a phenotype (9).

However, tissue repair is a finely orchestrated, multi-stage process, and Mp perform critical but contrasting functions in each stage. “Wound healing” is not a single well-defined function of Mp, but comprises its own spectrum of overlapping functions, including phagocytosis, cytokine and growth factor secretion, and matrix remodeling, all of which may be performed by Mp of different phenotypes. We argue below that nomination of IL-4 activated or M2a Mp as the primary Mp effectors of tissue repair is an oversimplification at best, and probably inaccurate. The actual *in vivo* phenotypes of Mp during the different phases of tissue repair have only recently begun to be characterized, and the factors regulating induction and modulation of Mp activation are poorly understood.

a. Transforming growth factor β

The notion that that IL-4/IL-13-induced M2a Mp are tissue repair Mp is based largely on their upregulation of TGFβ and arginase, as well as their ability to produce certain ECM components (9,51,61). IL-4-activated M2a Mp increase production of TGFβ (51), which is a powerful activator of fibroblast collagen production (62,63). It is likely that production of TGFβ by M2a Mp is at least

partly responsible for the enhancement of fibroblast proliferation and collagen production observed in Mp/fibroblast co-cultures (51). Interestingly, however, production of TGFβ by Mp during *in vivo* tissue repair does not appear to require the canonical M2a activators IL-4 or IL-13 (64). Therefore, M2a activation with IL-4 appears to be sufficient but not necessary for stimulation of Mp TGFβ production. TGFβ is a known mediator of fibrosis in numerous tissues (11,34,65,66), and also plays a critical role in skin wound healing through regulation of inflammatory cell recruitment, fibroblast activity, and keratinocyte migration (67). However, the relative importance of TGFβ production by Mp versus other cell types is uncertain.

b. Arginase

Rodent M2a Mp also express arginase, which has been suggested to be important for tissue repair (9,68). Importantly, in contrast to rodent Mp, human M2a Mp or monocytes do not express arginase (69,70). Rodent Mp activated *in vitro* with IL-4 increase mRNA and protein expression of arginase, accompanied by an increase in enzymatic activity (71). Arginine metabolism through the arginase pathway produces polyamines, which are important for cell proliferation (72), and proline, which is a major component of collagen. In culture, IL-4 or IL-13 increase proline production by Mp, and this is mediated by arginase (73). However, proline produced via the arginase pathway may not be a limiting factor for collagen synthesis (74), and the role of arginase in collagen production and fibrosis *in vivo* is complex and likely context-dependent (44,75,76). Arginase is

expressed during injury and repair in diverse models and tissues, including dystrophic skeletal muscle lesions (75), fibrotic liver (73), experimental glomerulonephritis (77), and human diabetic foot ulcers (78). However, arginase can be produced by cells other than Mp (74,77), and since human Mp do not produce arginase (69,70), human arginase appears to be derived entirely from non-Mp cell types (74). Additionally, arginase expression in cultured rodent Mp is increased not only by IL-4 but also by LPS (58,77). Thus, the presence of arginase, or even of arginase-expressing Mp, does not necessarily imply that IL-4/IL-13-induced M2a Mp are present in a tissue.

c. Extracellular matrix components

In addition to indirectly promoting fibroblast collagen synthesis via TGFβ and possibly arginase, M2a Mp are capable of directly synthesizing certain ECM components, including collagen type VI, fibronectin, and TGFβ-inducible gene (βIG)-H3 (79,80). The ECM-associated protein βIG-H3 promotes adhesion and migration of monocytes, keratinocytes, and fibroblasts (81-83), and increases fibroblast collagen production (84). βIG-H3 is expressed in numerous tissues in situations of injury or disease and can be expressed by many different cell types in addition to Mp (81-86). The relative importance of Mp versus other cell types, particularly fibroblasts, for production of ECM components during tissue repair is questionable.

d. Macrophage heterogeneity

Clearly, cultured M2a Mp produce factors that are known to be involved in tissue repair, and *in vivo* Mp in an actual wound can produce many of these same factors. Thus it may seem natural to conclude that M2a Mp perform tissue repair functions *in vivo*. However, the assumption implicit in this line of reasoning is that the presence of an M2a "marker" (i.e. something which is upregulated in Mp culture by IL-4) indicates that the Mp was in fact activated by IL-4 and/or shares other characteristics of cultured M2a Mp. This assumption is often false. For instance, in a mouse wound healing model, Mp expression of the M2a "marker" CD206 does not require IL-4 or IL-13 and does not correlate, negatively or otherwise, with TNFα production (64). Additionally, in freshly isolated human peritoneal Mp, cell surface expression of CD206 or the M2c "marker" CD163 does not correlate with expression of the M2-associated genes TGFβ, CCL18, or MMP-9 (87). Thus, while it is clear that treatment of cultured Mp with particular cytokines produces characteristic phenotypes, the presence of one or a few phenotypic markers *in vivo* does not demonstrate that the Mp was activated by the associated cytokine, or that other aspects of the phenotype induced by that cytokine will also be present. The true *in vivo* phenotypes of tissue repair Mp, as well as the factors which initiate and modulate these phenotypes, remain to be elucidated and are areas of active investigation.

4. Macrophage phenotypes during tissue repair and fibrosis

Recent studies have sought to characterize Mp phenotype *in vivo* during tissue repair, and to determine whether parallels can be drawn to *in vitro*-defined M1 and M2 phenotypes. An emerging dogma in tissue repair literature suggests that M1 Mp are the predominant population present during the first few days after injury, corresponding to the inflammatory and early proliferative phases, while M2a Mp are the primary effectors of later stages of repair, or the later proliferative and remodeling phases (7,8,88). If this is the case, then Mp would be expected to exhibit typical M1 markers during the early days after injury, while M2a markers would be expressed as repair progresses. For instance, Mp of the inflammatory and early proliferative phases would be expected to produce abundant inflammatory cytokines, while Mp at later stages would be expected to produce anti-inflammatory cytokines and express CD206 and other M2a surface markers. However, we argue here that while Mp of the inflammatory phase do in fact seem to present a more pro-inflammatory phenotype than their later phase counterparts, *in vivo* tissue repair Mp at any time point do not share sufficient similarities with *in vitro*-defined phenotypes to justify labeling them as wholly M1 or M2.

a. Pro-inflammatory cytokines

The pattern of pro-inflammatory cytokine production by tissue repair Mp is perhaps the strongest evidence for the early-M1/late-M2 paradigm, or at least for switching from a pro- to an anti-inflammatory phenotype. The M1 activator IFNγ

is rapidly upregulated after injury to skeletal muscle and skin, and is required for proper healing of these tissues (26,89-91). Similar to its effects on cultured Mp, IFNγ may promote an M1-like phenotype in Mp during muscular dystrophy (92). The M1-associated cytokines IL-1β, TNFα, IL-6, and IL-12 are expressed by Mp during the first few hours to days after acute injury in numerous tissues, including skin (42,64,91,93,94), liver (95), kidney (96), and skeletal muscle (26,39). In skin wounds, Mp exhibit decreased production of pro-inflammatory cytokines after the first week post-injury (42,64), though this time-dependent decrease remains to be confirmed in other tissues. Unpublished data from our laboratory suggest that mRNA expression levels of IL-1β and TNFα by Mp in damaged skeletal muscle at 3 days post-injury is similar to that of bona fide M1 Mp, and gradually declines as healing progresses (presented in this dissertation, and manuscript in preparation). However, Mp expression of TGFβ and IL-10 also appears to peak at this early time-point (presented in this dissertation, and manuscript in preparation). Similarly, at 48 hours after acute liver injury, Mp express IL-10 simultaneously with TNFα and IL-1β (95). This early expression of the anti-inflammatory cytokines TGFβ and IL-10 may suggest that even the early-invading Mp population is not purely M1.

b. Inducible nitric oxide synthase

In addition to pro-inflammatory cytokine production, expression of inducible nitric oxide synthase (iNOS) is a hallmark of M1 activation in rodent Mp (10). In contrast to the fairly consistent upregulation of pro-inflammatory

cytokines by early tissue repair Mp, expression of iNOS appears to be highly context-dependent. In kidney ischemia-reperfusion injury, Mp expression of iNOS is highest at 1 day post-injury and subsequently declines, consistent with an early M1 phenotype (96). However, Mp in incisional skin wounds appear to express iNOS only in response to invading pathogens and not as an inherent feature of the tissue repair response (64,97). After excisional skin wounding in rats (90) or photochemical liver injury in mice (98), only a fraction of the wound Mp express iNOS, and the heterogeneity of the Mp population may be dependent on proximity to the injured site (98). Studies from our own laboratory have produced contradictory data in two different injury models: skeletal muscle Mp express iNOS at 5 days after chemically-induced injury (26) but do not express iNOS at any time point after laceration injury (data presented in this dissertation, and manuscript in preparation). The reasons for this discrepancy remain to be determined, since Mp were obtained by similar methods, both injuries are expected to be sterile, and no evidence of infection was observed in either case. The varying expression of iNOS by Mp during tissue repair highlights the complexity of *in vivo* phenotypic regulation. Taken together, the pattern of inflammatory cytokine production and iNOS expression suggests a pro-inflammatory but not entirely M1 Mp phenotype during the early stages of tissue repair. Further studies will be needed to determine the factors that regulate this phenotype, and time-course studies will be needed to determine whether Mp expression of inflammatory cytokines decreases with time during repair of various tissues.

c. Anti-inflammatory cytokines

If Mp transition from an M1-like to an M2a-like phenotype as repair progresses, then anti-inflammatory cytokine expression by Mp would be expected to be low or absent during early stages of repair and elevated during the later stages. Indeed, during mouse skin wound healing, Mp expression of TGFβ increases over the first 7-10 days post-injury while pro-inflammatory cytokine production decreases (42,64), consistent with an M2a-like phenotype during the later stages of repair. In skeletal muscle, TGFβ and IL-10 are expressed at 4 days post-injury primarily by the Ly6C-negative Mp subset, which predominates during the later stages of repair (39); however, the time-course of cytokine expression was not specifically investigated in this study. Interestingly, unpublished data from our laboratory suggest that Mp expression of TGFβ and IL-10 peaks relatively early around 3 days after muscle laceration injury (presented in this dissertation, and manuscript in preparation), contrary to expectations of an early-M1 to late-M2 transition. Furthermore, levels of IL-10 and TGFβ expression are not necessarily co-regulated; Mp display increased expression of IL-10 but not TGFβ during chronic liver fibrosis, and deficiency of the chemokine receptor CX_3CR_1 results in reduced IL-10 but increased TGFβ expression by Mp (99). Thus, even if particular M2a-associated cytokines are expressed by Mp during tissue repair, this does not guarantee that other aspects of the M2a phenotype are present.

Additionally, the canonical M2a activators IL-4 and IL-13 may not always be required for Mp activation during tissue repair. IL-4 and IL-13 are not detected in mouse skin wound cells or fluids at any time, and deletion of the common receptor IL-4Rα has no effect on Mp phenotype at 7 days post-injury, indicating that despite their predominantly anti-inflammatory cytokine profile, these cells are not bona fide M2a Mp (64). IL-4 is also not detectable in mouse incisional or excisional skin wounds at either early or late time-points (42,93), but is present in mouse peritoneal incisions (100), rat excisional skin wounds (90), human dermal wounds (91), and infarcted rat myocardium (101). The reasons for and consequences of this differential expression of IL-4 remain unclear. Further studies will be needed to determine whether IL-4 and IL-13 are involved in Mp activation in different tissues, and to categorize the time course of anti-inflammatory cytokine expression by Mp during tissue repair. Taken together, existing data on anti-inflammatory cytokine production suggest that Mp may become more M2a-like as repair progresses, though this transition may not happen in all situations. Additionally, because IL-4/-13 activation is not required for upregulation of IL-10 and TGFβ, the presence of other aspects of the M2a phenotype should not be inferred from cytokine expression alone.

d. M2a “markers”

In addition to anti-inflammatory cytokine production, M2a Mp are characterized by expression of phenotypic “markers” such as CD206, Ym1, dectin-1, and FIZZ1 (58). If Mp undergo a switch from an M1-like to an M2a-like

phenotype during tissue repair, Mp expression of these M2a markers would be expected to increase as repair progresses. Indeed, in mouse and human skin wounds and in injured mouse kidney, Mp expression of CD206 increases during the later stages of repair (42,64,94,96). However, although CD206, Ym1, and dectin-1 are expressed by skin wound Mp at 1, 3, and 7 days post-injury (64), these three M2a-associated proteins exhibit entirely different temporal patterns: CD206 increases somewhat as repair progresses, while Ym1 is expressed most highly at 1 day post-injury and then decreases, and dectin-1 expression remains similar among blood monocytes and wound Mp at all time points (64). Additionally, CD206 expression is similar among TNFα-high, -low, or -negative Mp (64), which would not be expected if distinct M1 and M2a populations were present. Similarly, in skin wound Mp, CD206 expression does not appear to correlate, negatively or otherwise, with expression of Ly6C (42), which is sometimes associated with a pro-inflammatory phenotype (39,57,102). Collectively, data from these studies indicate that the M2a “markers” CD206, Ym1, and dectin-1 can be regulated independently of each other in skin wound Mp, and can be co-expressed with M1-associated or pro-inflammatory genes and proteins. Thus, Mp during skin wound healing exhibit a heterogeneous and temporally regulated phenotype that does not conform strictly to M1/M2 categorization (64).

In other tissues, M2a markers are often present, but most studies have not determined whether these markers are expressed by Mp or by other cell types, and any conclusions drawn regarding Mp phenotype are therefore dubious.

Seven days after photochemical injury to the liver, CD206+ cells are observed near the border of the injured site (98), though these cells were not specifically verified as Mp and no other time-points were examined. Similarly, after peritoneal incisional wounding, peritoneal exudate cells transiently express elevated Ym1 and FIZZ1 mRNA, though this cell population contains lymphocytes and neutrophils in addition to Mp (100), and each of these cell types could express one or both genes (103,104).

e. Summary of *in vivo* macrophage phenotypes

In summary, existing evidence is limited but does not support the notion that Mp present during tissue repair are bona fide M2a Mp. An M1-like to M2a-like transition may indeed occur in some situations, though this appears to be context-dependent, and the co-expression of M1- and M2a- associated markers precludes classification of either early or late Mp as purely M1 or M2a [Table I]. Given the growing belief that M2a Mp participate in tissue repair, surprisingly few studies have actually examined Mp-specific expression of M2a-associated genes or proteins *in vivo*. Whole-tissue content of cytokines or other markers of M2a activation is insufficient to determine the phenotype of Mp during *in vivo* tissue repair, since whole-tissue levels could be affected by expression in non-Mp cells or by changes in the number of Mp in the tissue. Additionally, a comprehensive *in vivo* Mp phenotype cannot be deduced solely from a limited set of phenotypic markers, since different M2a-associated genes or proteins can exhibit entirely different temporal regulation and can be co-expressed with M1-associated

markers (42,45,58,64,105). Further studies of Mp phenotype during tissue repair are essential, but we suggest that investigation of specific functions (such as phagocytosis or stimulation of stem cell proliferation and differentiation) of Mp isolated from tissues undergoing repair may be more fruitful than attempts to sort tissue repair Mp into discrete *in vitro*-defined subsets that may not exist *in vivo* and are based on markers that may have no direct role in tissue injury and repair.

TABLE I. SUMMARY OF *IN VITRO* AND *IN VIVO* MACROPHAGE PHENOTYPES

	Cultured Mp		***In Vivo* Tissue Repair Mp**	
	M1	**M2a**	**Early**	**Late**
Activated by:	IFNγ, TNFα, LPS	IL-4, IL-13	Poorly defined DAMPs. IFNγ may promote M1-like polarization (skeletal muscle). IL-4 may or may not be present.	Not well known, possibly phagocytosis. IL-4 may or may not be present. IL-4/-13 do not appear to be required for activation (skin).
Pro-inflammatory cytokines	IL-1β, TNFα, IL-6, IL-12	Low	IL-1β (skin, liver, skeletal muscle), TNFα (skin, liver, skeletal muscle), IL-6 (skin), IL-12 (kidney)	Decrease with time (skin, skeletal muscle). IL-12 also decreases with time in kidney.
iNOS	High (only in rodent)	Low	Context-dependent. May be related to host-defense, not tissue repair.	Decreases with time (kidney). If present, time course not known in other tissues.
Anti-inflammatory cytokines	Low	TGFβ high, IL-10 low	TGFβ (skeletal muscle, skin) and IL-10 (liver & skeletal muscle, may not be present in skin)	TGFβ increases with time (skin). IL-10 time course not known.
CD206	Low	High	Present (skin)	Increase with time (skin)
Dectin-1	Low	High	Present (skin)	No change with time (skin)
Ym1	Low	High	High (skin)	Decrease with time (skin)

5. Macrophage-based therapies for tissue repair

Though endogenous Mp during tissue repair do not appear to conform to *in vitro*-defined M1 and M2 categories, knowledge about the regulation and function of different Mp phenotypes has the potential to yield new therapies for improving healing. Because cultured M2a Mp produce many factors important for tissue repair, treatments that promote a more M2a-like phenotype in endogenous Mp may have potential to promote healing. Conversely, during tissue fibrosis, skewing endogenous Mp towards a more M1-like phenotype may help to suppress fibroblast activity or enhance production of matrix-degrading enzymes. Alternatively, cell therapy with *ex vivo*-activated Mp may be able to accomplish similar goals.

a. Modulation of macrophage phenotype

While these potential therapies remain largely hypothetical, a number of animal studies have provided proof-of-concept for Mp-based interventions, and some progress has even been made in treatment of human wounds. In patients with inflammatory bowel disease given anti-TNFα treatment, improved mucosal healing is associated with an increased percentage of CD206-positive Mp, though this study lacked an untreated control group (106). Similarly, after mouse spinal cord injury, blockade of the IL-6 receptor promotes regeneration and recovery of locomotor function while skewing endogenous Mp toward a more M2a-like phenotype (107). An M2a bias may also be beneficial in the *mdx* mouse model of muscular dystrophy, in which genetic deletion of IFNγ ameliorates the

disease while increasing whole-muscle expression of M2a-related genes (92). IFNγ also participates in Mp polarization during repair of acute skeletal muscle injuries (26); however, in contrast to chronic *mdx* pathology, disruption of IFNγ is in this case detrimental to healing (26), suggesting that the ideal repair-promoting Mp phenotype may differ based on the underlying pathology.

b. Macrophage cell therapy

In addition to phenotypic manipulation of endogenous Mp, a number of studies have indicated that cell therapy with exogenous Mp can promote healing in a variety of tissues, and prior *ex vivo* activation of these Mp to an M1 or M2a phenotype may alter their ability to promote repair. In a mouse model of kidney injury, intravenous delivery of M2a Mp reduces injury and fibrosis, while exogenous M1 Mp exacerbate the disease (96,108). Similarly, in a mouse model of type I diabetes, transfusion of M2a Mp reduces pancreatic injury and kidney fibrosis, while non-activated Mp have no effect (109). *Ex vivo* activation may not be necessary for Mp therapy to be effective in other tissues, since naïve bone marrow-derived Mp reduce liver fibrosis and enhance regeneration after carbon tetrachloride injury in mice (110), and application of peritoneal Mp to skin wounds of young or old mice accelerates their closure (111).

While animal studies have provided proof-of-concept for Mp cell therapy in repair of various tissues, doubts have been raised as to the feasibility and cost-effectiveness of its use in humans (112,113). To assist in clinical translation of Mp cell therapy, Danon and colleagues have developed a method for obtaining

activated monocyte/Mp, together with other cell types, from whole-blood units (113,114). These hypoosmotically activated cell suspensions enhance healing of ulcers in elderly and diabetic patients (113,115), improve cardiac function and scar remodeling in infarcted rat hearts (114), and reduce mortality and duration of hospitalization in patients with infected sternal wounds after cardiac surgery (116). Hypoosmotic activation increases expression of IL-1β and IL-6 and enhances phagocytosis to a similar or greater degree than LPS stimulation (117); however mRNA expression of TGFβ and IL-4 is also increased (118), indicating that hypoosmotic activation is not entirely similar to M1 stimulation.

c. Summary of macrophage-based therapies

In summary, cell therapy with exogenous Mp has proven beneficial to healing of a variety of tissues, and *ex vivo* activation of these Mp to an M1, M2, or other phenotype can alter their efficacy (96,108,109,113-116). Similarly, manipulation of endogenous Mp phenotype holds promise as a potential tissue repair therapy. Further studies comparing the efficacy of different modes of Mp activation, along with an improved understanding of the phenotypic fate of exogenous Mp, may help to optimize Mp-based therapies for healing of different tissues and bring these promising treatments closer to widespread clinical reality.

6. Macrophage phenotypes in tissue repair: summary and conclusions

Mp are critical orchestrators of repair and regeneration in numerous tissues, but may also contribute to chronic tissue damage and fibrosis. Different stimuli can produce a broad spectrum of functional Mp phenotypes, and this plasticity likely contributes to the seemingly contradictory roles of Mp in tissue injury, regeneration, and fibrosis. The plethora of factors that regulate Mp activation also complicate identification of Mp phenotypes *in vivo*; tissue repair Mp are exposed to a vastly more complex microenvironment than Mp treated with one or a few cytokines in a cell culture dish. Therefore, it is not surprising that *in vivo* tissue repair Mp do not conform to existing *in vitro*-defined phenotypic categories. While Mp during the early stages of tissue repair seem to present a more generally pro-inflammatory profile than their later counterparts, early Mp may lack iNOS expression or may express CD206, Ym1, and IL-10 in addition to pro-inflammatory cytokines, indicating that these early repair Mp do not exhibit an entirely M1 phenotype. Similarly, while later Mp populations may increase expression of some M2a-associated markers such as CD206, others such as Ym1 may be oppositely regulated, and the canonical M2a activators IL-4 and IL-13 may or may not be present depending on the tissue and mode of injury. These data indicate that Mp during the later stages of tissue repair are not entirely of the M2a phenotype. In short, tissue repair Mp exhibit complex and heterogeneous phenotypes that change throughout the repair process and do not correspond to existing *in vitro*-defined categories.

Many open questions remain regarding the role of Mp in tissue repair. While Mp are known to promote tissue repair functions such as wound debridement, cell proliferation, angiogenesis, and matrix remodeling, much remains to be learned about the mechanisms by which Mp accomplish these functions. Similarly, while many potential modulators of Mp activation have been identified *in vitro*, the actual factors that regulate Mp phenotype during tissue repair remain to be elucidated. Furthermore, whether Mp of different phenotypes during the inflammatory, proliferative, and remodeling phases are derived from sequential invasion of distinct blood monocyte populations or from a change in phenotype of existing wound Mp is under debate (39,119). Finally and perhaps most importantly, rodent and human Mp do not exhibit entirely similar responses to *in vitro* activation (9,74,120,121), and it remains to be determined whether there are also species-dependent differences in Mp function during *in vivo* tissue repair. An improved understanding of the reciprocal regulation of Mp phenotype and the tissue repair environment will provide insight into novel therapies based on manipulating the function of endogenous Mp or using exogenous Mp as cell therapy to promote healing.

CHAPTER II

MACROPHAGE ACTIVATION AND SKELETAL MUSCLE HEALING FOLLOWING TRAUMATIC INJURY

A. INTRODUCTION

Skeletal muscle injuries are among the most common injuries suffered during sporting activities, military combat, and accidents of daily life. Repair of the muscle after traumatic injury or exercise-induced tears is prolonged and incomplete. Return to pre-injury activity levels often takes months, while injured muscle tissue is replaced by fibrotic scar tissue, which persists indefinitely (20). Muscle force production is decreased due to the non-contractile nature of the scar, and the scar site is highly susceptible to re-injury (20). In order to develop improved treatments for traumatic muscle injuries, a better understanding of the mechanisms underlying both muscle regeneration and fibrosis is necessary.

1. Overview of skeletal muscle repair

Repair of skeletal muscle and other tissues occurs in overlapping phases of inflammation, proliferation, and remodeling (22). Within hours after injury, inflammatory cells invade the injured muscle, where they clear necrotic tissue and release soluble mediators that promote regeneration. Meanwhile, muscle satellite cells are activated, proliferate, and fuse to form new myofibers, which enlarge and mature to restore tissue structure and function. Simultaneously, however, production of extracellular matrix (ECM) proteins by fibroblasts can result in formation of a fibrous scar at the site of injury.

2. Macrophages in muscle repair

Numerous studies have demonstrated that macrophages (Mp) are essential for skeletal muscle regeneration. Depletion of Mp after toxin-induced muscle injury impedes clearance of necrotic tissue and reduces the number and diameter of regenerating muscle fibers (3,14,39). In addition to their classical function of phagocytosing dead tissues, Mp may actively regulate skeletal muscle regeneration by modulating myoblast activity. In *in vitro* experiments, cell-cell contacts between Mp and myoblasts enhance survival of both cell types (16). Mp also produce soluble factors that enhance myoblast proliferation (16,17). Additionally, Mp can promote proliferation, differentiation, and/or fusion of myoblasts (39). However, Mp can also promote fibrosis in numerous tissues (5,105,108,122) and can either increase or decrease collagen production by cultured fibroblasts (51).

3. Macrophage plasticity *in vitro*

The diverse functions of Mp in tissue repair may be explained by the remarkable plasticity of these cells. Mp exhibit diverse and often opposing phenotypes when exposed to different environmental stimuli. Inflammatory stimuli such as bacterial components or IFNγ produce a “classically activated” Mp phenotype, also called “M1” (10). M1 Mp produce large amounts of reactive oxygen and nitrogen species and pro-inflammatory cytokines, and are effective antigen presenters and T cell activators. In contrast to the M1 state, Mp also

exhibit a spectrum of non-inflammatory, “alternatively activated” phenotypes, also referred to as “M2” activation (9,10). One of the most well described M2 phenotypes, known as “M2a,” results from stimulation with interleukin (IL)-4 or IL-13. M2a Mp express high levels of TGFβ, as well as the mannose receptor CD206 and the scavenger receptor CD36 (47,51,123).

M2a Mp have been implicated in fibrosis of numerous tissues, including lung (124), liver (125), and adipose tissue (105). Additionally, M2a Mp are thought to contribute to development of fibrosis in muscular dystrophy (62,75). In culture, M2a Mp enhance fibroblast collagen synthesis through production of TGFβ (51). Additionally, M2a Mp express high levels of arginase, which may steer arginine metabolism towards collagen production (9). Conversely, M1 Mp may have anti-fibrotic properties, possibly by reducing fibroblast collagen production or increasing collagen degradation (51). M1 Mp express collagen-degrading matrix metalloproteases as well as iNOS, which may divert arginine metabolism away from collagen synthesis and towards NO production. However, most of our current knowledge of Mp phenotype is derived from *in vitro* studies, and an M1 or M2a phenotype of Mp *in vivo* is frequently extrapolated based on expression of a small set of phenotypic “markers” (76,90,96,98,126). While M1 and M2a Mp can certainly modulate fibroblast activity in culture, convincing evidence that Mp of a strictly M1 or M2a phenotype are present during tissue repair *in vivo* is lacking.

4. Macrophage phenotypes during *in vivo* muscle repair

The role of the various Mp phenotypes in skeletal muscle repair has recently begun to be elucidated. After toxin-induced muscle injuries that result in good regenerative outcomes, invading Mp transition from a pro-inflammatory phenotype to an anti-inflammatory phenotype over the course of the healing process, then disappear from the muscle as healing progresses (14,39). Whether Mp during the different stages of muscle healing correspond strictly to *in vitro*-defined M1 and M2 phenotypes remains to be determined. *In vitro* studies demonstrate that M1 Mp can clear necrotic tissue by phagocytosis and promote proliferation of myoblasts (39). In turn, phagocytosis of muscle debris triggers a switch to a more anti-inflammatory, M2a-like phenotype, which promotes myoblast differentiation and fusion (39). In mice, Mp depletion after toxin-induced injury significantly impairs regeneration (3,14,39); early depletion of Mp impairs necrotic tissue clearance and greatly reduces the number of regenerating myofibers, while depletion of Mp during the later stages of healing reduces regenerating myofiber diameter (39). However, little is known about the contribution of Mp to repair of exercise- or trauma-induced muscle injuries, which are more representative of common human injuries and which result in persistent fibrous scars. Whether Mp exhibit a switch from pro- to anti-inflammatory phenotype following traumatic muscle injury is not known, and the ability of M1 or M2a Mp to inhibit or promote scar formation after muscle injury has not been investigated.

5. Hypothesis and summary of results

We hypothesized that fibrosis after traumatic skeletal muscle injury would be accompanied by prolonged Mp accumulation, and that these Mp would transition from an early M1 phenotype to a later M2a phenotype. We also hypothesized that cell therapy with exogenously activated M1 Mp would enhance regeneration and reduce fibrosis. However, while persistent Mp accumulation was observed after laceration injury, Mp did not exhibit either of the canonical *in vitro*-defined phenotypes. Instead, Mp upregulated both M1 and M2a markers early after injury, followed by an apparent deactivation. Importantly, treatment of injured muscles with exogenous M1 Mp reduced fibrosis but failed to enhance regeneration.

B. MATERIALS AND METHODS

1. Animals

Wild-type C57BL/6 mice were obtained from Harlan (Indianapolis, IN) and bred in our animal facility. Mice were housed at 22-24°C on a 12:12h light-dark cycle and provided with food and water ad libitum. All experiments were performed on mice aged 10-15 weeks. The Animal Care Committee at the University of Illinois at Chicago approved all experimental procedures.

2. Muscle injury

Bilateral laceration of the gastrocnemius muscles was performed as adapted from a previously described protocol (23). Briefly, mice were anesthetized with ketamine (100mg/kg) and xylazine (5mg/kg), and a1cm longitudinal incision was made on the posterior hindlimb to expose the gastrocnemius muscle. A scalpel was used to lacerate the muscle transversely at its widest point through the entire thickness of the lateral half of the muscle, taking care to preserve the central neurovascular complex. The lacerated muscle was then placed back in the muscle bed, the skin was closed with 6-0 nylon suture, and the procedure was repeated on the contralateral leg. Mice were allowed normal cage activity during recovery. At various time points after injury, mice were anesthetized and sacrificed by cervical dislocation, and gastrocnemius muscles were collected for histology or Mp isolation.

3. Histology: identification of injury site

Gastrocnemius muscles were embedded in freezing medium and flash frozen in 2-methylbutane cooled on dry ice. Serial transverse 10μm-thick cryosections were taken throughout the entire injured/regenerating portion of the muscle, and sections with the greatest percentage of damaged, non-regenerated area were selected for further analysis by staining with hematoxylin and eosin, Masson's Trichrome, or immunohistochemistry.

4. Histology: regeneration

Regeneration was quantified in hematoxylin and eosin-stained sections by morphological analysis on five representative images of each muscle section obtained using a 40x objective on an Eclipse 80i microscope with DS-Fi1 camera and NIS-Elements BR software (Nikon, Melville, NY). Normal or regenerating fibers were identified as those containing peripherally or centrally located nuclei, respectively, without evidence of damage. Percent normal area was quantified as the sum of cross-sectional areas of all normal fibers, divided by the total area of the image. Percent regenerating area was quantified as the sum of cross-sectional areas of all regenerating fibers, divided by the total area of the image. Percent damaged, non-regenerated area was calculated as 100% minus percent regenerating area minus percent normal area, i.e. the percent area not occupied by either normal or regenerating muscle fibers.

5. Histology: collagen accumulation

Masson's Trichrome staining was performed, and three to six 20x images were taken of the injured site in each muscle. Masson's Trichrome stains muscle fibers red, nuclei black, and collagen blue. Collagen accumulation was quantified as the percent of the total image area that was stained blue.

6. Immunohistochemistry

Mp and neutrophils were identified in muscle cryosections by standard immunohistochemical methods. Briefly, sections were fixed in cold acetone,

quenched with 0.3% hydrogen peroxide, and blocked with buffer containing 3% BSA. Sections were then incubated overnight with primary antibodies against F4/80 (rat anti-mouse, Serotec, Oxford, UK) or Ly6G (rat anti-mouse, BD Biosciences, San Diego, CA). Next, sections were incubated with biotinylated anti-rat secondary antibody (Vector Laboratories, Burlingame, CA) followed by avidin D-horseradish peroxidase (Vector Laboratories), and developed with a 3-amino-9-ethylcarbazole kit (Vector Laboratories). Mp or neutrophil accumulation was quantified as percent F4/80 or Ly6G-stained area, respectively, in six 20x images per muscle.

7. Muscle macrophage isolation

Gastrocnemius muscles were digested and Mp isolated by magnetic separation using a modification of a previously described protocol (127). Briefly, the lateral heads of both gastrocnemius muscles were dissected, minced, and digested in 0.67mg/ml each of collagenase type 1, collagenase type XI, and hyaluronidase (Sigma-Aldrich, St. Louis, MO) in DMEM (Invitrogen, Carlsbad, CA). Cell suspensions were then filtered through 70μm mesh, centrifuged, and resuspended in MACS buffer (0.5% FBS and 2mM EDTA in PBS, pH 7.2). Neutrophils, T-cells, and B-cells were depleted using FITC-conjugated anti-Ly6G, anti-CD3, and anti-CD19 (Biolegend, San Diego, CA), followed by an anti-FITC antibody conjugated to magnetic beads (Miltenyi Biotec, Auburn, CA). The cell suspension was run through a magnetic column (Miltenyi Biotec) following the manufacturers instructions. From the Ly6G/CD3/CD19-negative fraction, Mp

were then isolated by positive selection using an anti-CD11b antibody conjugated to magnetic beads (Miltenyi Biotec). This suspension was run through another magnetic column, and Mp were obtained as the CD11b-positive, Ly6G/CD3/CD19-negative fraction. Mp were either plated immediately for IL-10 secretion or stored at -80°C for RNA isolation, as described below.

8. Culture and activation of bone marrow-derived macrophages

Bone marrow derived Mp were cultured as previously described (14). Briefly, bone marrow was flushed from mouse femurs and tibias using DMEM supplemented with 10% heat-inactivated FBS, 10% L929 cell-conditioned medium (source of M-CSF), 2mM L-glutamine, and 1% penicillin/streptomycin (Sigma-Aldrich). After 4 days, cells cultured in this manner are >95% F4/80+ by flow cytometry (14). Day 6 cultures were treated with 10ng/ml each IFNγ and TNFα, or 10ng/ml IL-4, or 10ng/ml IL-10 (Peprotech, Rocky Hill, NJ) to obtain M1, M2a, and M2c Mp, respectively. After 24h of cytokine exposure, Mp were harvested for RNA isolation or Mp cell therapy.

9. RNA isolation and real-time PCR

Total RNA was isolated from muscle-derived Mp (i.e. magnetically separated CD11b-positive, Ly6G/CD3/CD19-negative cells) and bone marrow derived Mp using the RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Equal amounts of RNA were reverse-transcribed using the Thermoscript RT-PCR system (Invitrogen), and real-time PCR was

performed in a 7500Fast System (Applied Biosystems, Carlsbad, CA) using TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assay primer/probe sets (Applied Biosystems) for mouse IL-1β, TNFα, inducible nitric oxide synthase (iNOS), CD36, CD206, IL-10, TGFβ, and GAPDH. Relative expression was determined using the $2^{-\Delta\Delta CT}$ method (128). Expression of M1-associated genes (IL-1β, TNFα, iNOS) was normalized to levels expressed by *in vitro* activated M1 Mp, and expression of M2a-associated genes (CD206, CD36, IL-10, and TGFβ) was normalized to levels expressed by *in vitro* activated M2a Mp. GAPDH was used as endogenous control gene; preliminary experiments with other candidate control genes demonstrated that GAPDH was most stable both within and across experimental groups compared to β-actin, TBP, or β-2 microglobulin (not shown).

10. Interleukin-10 secretion

Muscle-derived Mp (i.e. magnetically separated CD11b-positive, Ly6G/CD3/CD19-negative cells) were plated in 96-well plates at 75,000 cells per well in DMEM supplemented with10% heat-inactivated FBS, 2mM L-glutamine, and 1% penicillin/streptomycin (Sigma-Aldrich). Mp from multiple mice were pooled to obtain sufficient cells from uninjured muscles. After 20 hours, conditioned media were collected and centrifuged, and the supernatant was analyzed for IL-10 using the Mouse IL-10 ELISA Ready-SET-Go kit (eBioscience, San Diego, CA), following the manufacturer's instructions.

11. Flow cytometry

The total population of cells present in muscle following laceration injury were isolated via enzymatic digest as described above. Fcγ receptors were blocked with purified anti-mouse CD16/CD32 (BD Biosciences), and extracellular antigens were labeled with FITC-, Alexa488-, or PE-conjugated antibodies against F4/80 (BioLegend), CD206, or CD36 (Serotec), or with non-specific isotype controls. Cells were then fixed and permeabilized using the BD Cytofix/Cytoperm Plus kit (BD Biosciences), and intracellular antigens were labeled with PE-, PerCP-Cy5.5-, or APC-conjugated antibodies against TGFβ, TNFα (BioLegend), or IL-10 (eBioscience), or with non-specific isotype controls. Stained cells were analyzed using an Accuri C6 flow cytometer and CFlow Plus software, version 1.0.227.4 (BD Biosciences). Mp were defined as F4/80-positive cells.

12. Macrophage cell therapy

Gastrocnemius muscles were lacerated and bone marrow Mp were grown as described above. Injection of M1 Mp , non-activated Mp, or PBS vehicle was performed at 7 days post-laceration. PBS vehicle was used to control for effects of injection, and non-activated Mp were used to determine whether M1 activation was necessary for Mp therapy to be effective. Briefly, Mp were washed three times and resuspended in PBS. Mp or PBS vehicle were delivered to the injured gastrocnemius in two injections of 25μL each. A total of $0.5x10^6$, $1x10^6$, or $2x10^6$ Mp were injected. After an additional 7 days (14 days post-injury), muscles were

collected for histological analysis of regeneration and fibrosis, as described above.

13. Statistical analysis

Statistical analysis was performed using SigmaPlot version 12.0 (Systat Software, San Jose, CA). Data that passed tests of normality and equal variance were analyzed by Student's t-test for two-group comparisons, or by one-way ANOVA and Student-Newman-Keuls post-hoc method for multiple comparisons. These data are presented as bar charts showing mean +/- standard deviation. Data that did not pass tests of normality and equal variance were analyzed by ANOVA on ranks with Dunn's post-hoc method. For more accurate portrayal of variability, non-normally distributed data (i.e. gene expression and IL-10 secretion, Figure 3) are presented in box-and-whisker plots with center line as median, boxes representing the 25^{th} and 75^{th} percentiles, whiskers representing the 10^{th} and 90^{th} percentiles, and outliers as dots. For all data, $p<0.05$ was taken to indicate statistical significance.

C. RESULTS

1. Muscle laceration heals by both regeneration and fibrosis

Consistent with previous studies (30,37), lacerated gastrocnemius muscle healed by a combination of regeneration and fibrosis. At 3 days post-laceration, the injured site was largely comprised of loose granulation tissue and

degenerating myofibers [Figure 2A & E]. By 7 days post-injury, centrally nucleated regenerating myofibers began to appear. The percent of total muscle area occupied by regenerating myofibers increased from 7 to 14 days post-injury, with a corresponding decrease in damaged, non-regenerated area. Subsequently, at 21 and 28 days post-injury, there was a gradual increase in percent area composed of mature, peripherally nucleated myofibers and a corresponding decrease in regenerating area. However, the percent damaged, non-regenerated area did not significantly decrease below ~40% after 14 days post-injury, indicating incomplete regeneration [Figure 2A]. This contrasts with healing after toxin-induced muscle injury, which results in complete regeneration (14,24,25). Furthermore, after 14 days, injured muscles contained a larger total number of myofibers versus uninjured [Figure 2B]. However, the average cross sectional area of individual myofibers failed to increase with time [Figure 2C], remaining at ~30% of the average uninjured myofiber size. Additionally, collagen accumulation was evident by 14 days post-injury, and this fibrosis persisted for at least 28 days [Figure 2D & E].

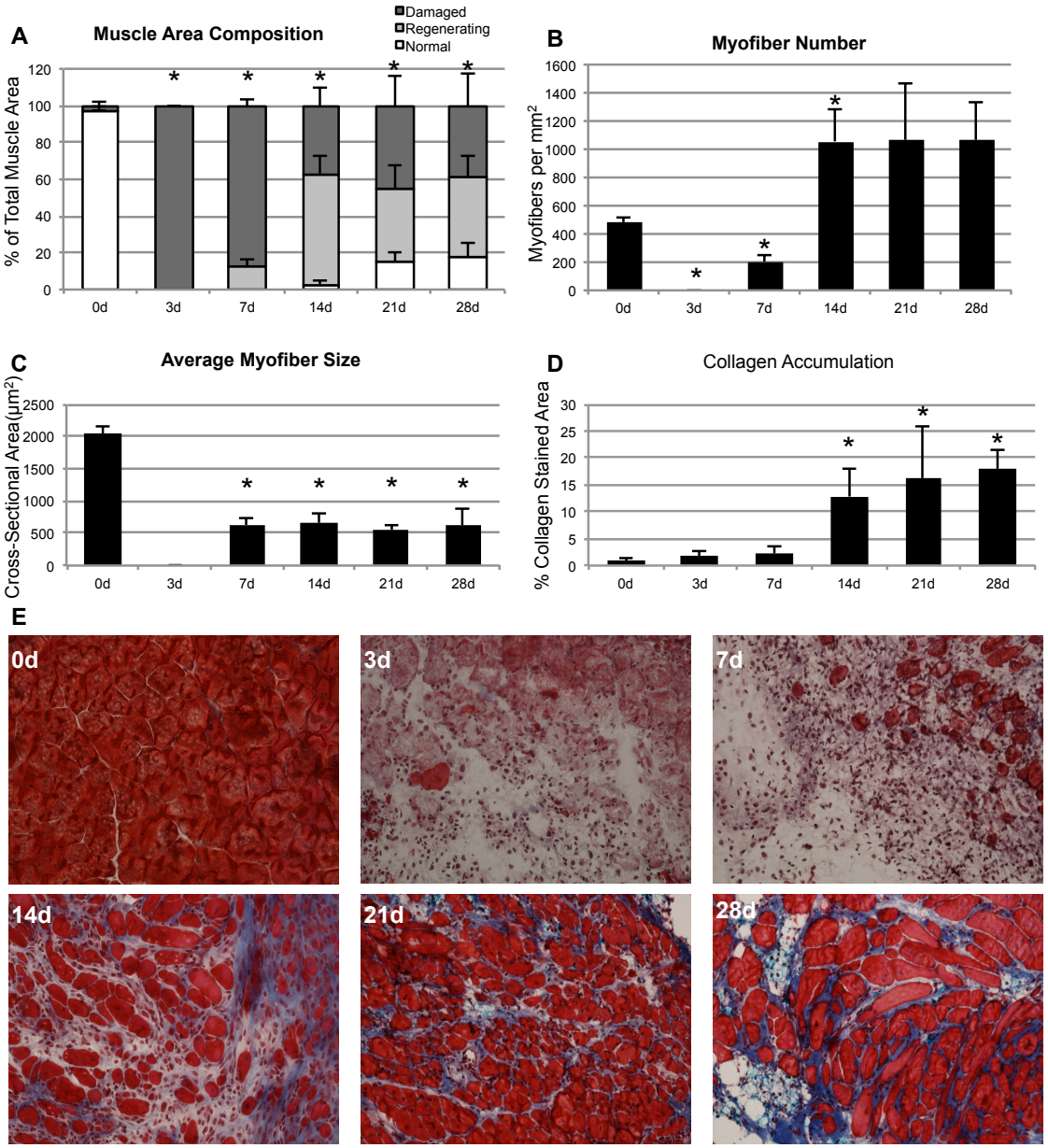


Figure 2. Lacerated muscle heals by both regeneration and fibrosis. Gastrocnemius muscles were lacerated, collected at the indicated time-points, and regeneration and fibrosis were quantified as described in Materials and Methods. Uninjured muscle (0d) served as control. (A) Percent of total cross-sectional area occupied by centrally nucleated ("Regenerating") or peripherally nucleated ("Normal") myofibers. "Damaged" area was defined as area not occupied by either type of myofiber. (B) Number of myofibers per mm^2. (C) Average cross-sectional area of individual myofibers. (D) Collagen accumulation. (E) Representative images of trichrome stained sections. Data are presented as mean +/- SD. * $p<0.05$ versus uninjured. In (A), * indicates significance for percent damage area.

2. Muscle laceration results in persistent macrophage accumulation, but these macrophages are not of M1 or M2a phenotype

While Mp are necessary for muscle regeneration, prolonged Mp accumulation and M2a bias are associated with fibrosis in numerous tissues (62,75,105,124,125). We hypothesized that muscle fibrosis after laceration injury would be accompanied by persistent accumulation of Mp. After injury, Mp accumulation was elevated by 3 days post-injury, peaked around 7 days, and subsequently declined [Figure 3A]. However, Mp accumulation remained elevated over uninjured levels for at least 21 days post-injury. In contrast, neutrophil accumulation peaked around 3 days post-injury, then rapidly returned to uninjured levels [Figure 3B].

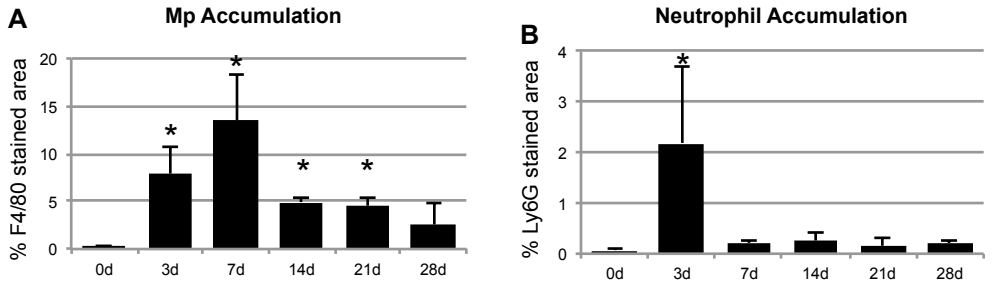


Figure 3. Muscle laceration results in prolonged accumulation of Mp but not neutrophils. Gastrocnemius muscles were lacerated, collected at the indicated time-points, and analyzed by immunohistochemistry as indicated in Materials and Methods. Uninjured muscles (0d) served as controls. (A) Mp accumulation was quantified as percent F4/80-stained area. (B) Neutrophil accumulation was quantified as percent Ly6G-stained area. Data are presented as mean +/- SD. * $p<0.05$ versus uninjured.

We also hypothesized that Mp would transition from an early M1-like to a later M2a-like phenotype during the course of muscle healing. To this end, we isolated Mp from injured and uninjured muscles by magnetic separation and examined expression of M1 and M2a-associated genes. Compared to resident Mp isolated from non-injured muscle, expression of the M1-associated cytokine IL-1β was elevated in Mp from 3 day injured muscles and subsequently declined towards uninjured levels [Figure 4A]. TNFα also showed a non-significant trend for increased expression at 3 days [Figure 4B]. Mp expression of both of these cytokines at 3 days post-injury was comparable to that of *in vitro* activated M1 Mp. In contrast, iNOS expression was low or undetectable in muscle Mp at all time points, despite robust expression in cultured M1 Mp [Figure 4C]. Contrary to expectations of an M1-to-M2a transition, expression of the M2a marker CD206 was not elevated over resident Mp expression at any time point after injury, and was expressed at low levels by all muscle Mp compared to *in vitro* M2a positive controls [Figure 4D]. Furthermore, Mp expression of the M2-associated genes CD36, TGFβ, and IL-10 peaked relatively early at 3 days post-injury [Figure 4E-G].

In contrast to the relatively modest upregulation of CD36 and TGFβ in muscle Mp after injury, median Mp expression of IL-10 at 3 days post-injury was elevated approximately 20- and 50-fold over *in vitro* M2c and M2a positive controls, respectively, and over 300-fold versus resident muscle Mp expression [Figure 4G]. To determine whether this powerfully anti-inflammatory cytokine was also upregulated at the protein level, we isolated Mp from injured and uninjured

muscles by magnetic separation and measured cytokine secretion into conditioned medium. Resident Mp from uninjured muscle did not secrete detectable amounts of IL-10. However, IL-10 secretion was markedly elevated in Mp from 3 day injured muscle, and declined by 7 days post-injury [Figure 4H]. Interestingly, *in vitro*-activated M2a and M2c bone marrow-derived Mp did not secrete detectable amounts of IL-10 (not shown).

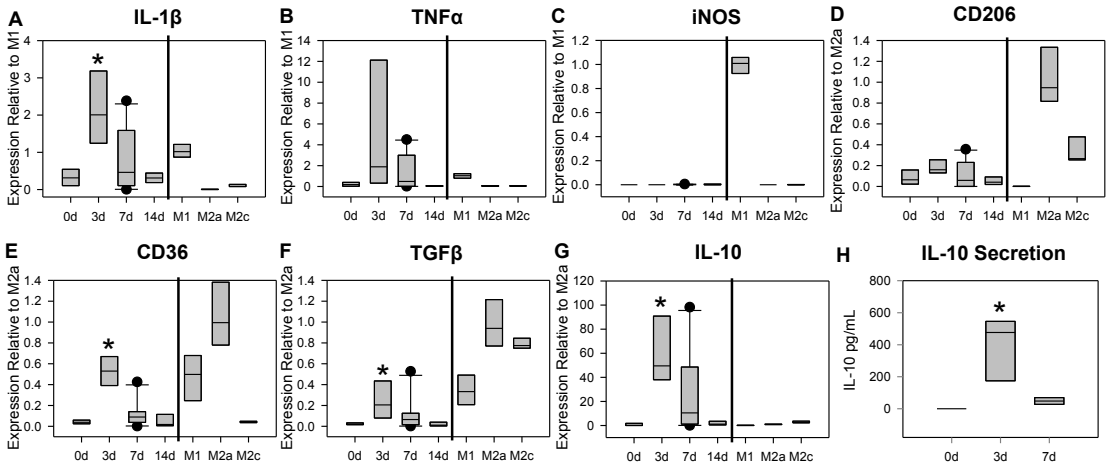
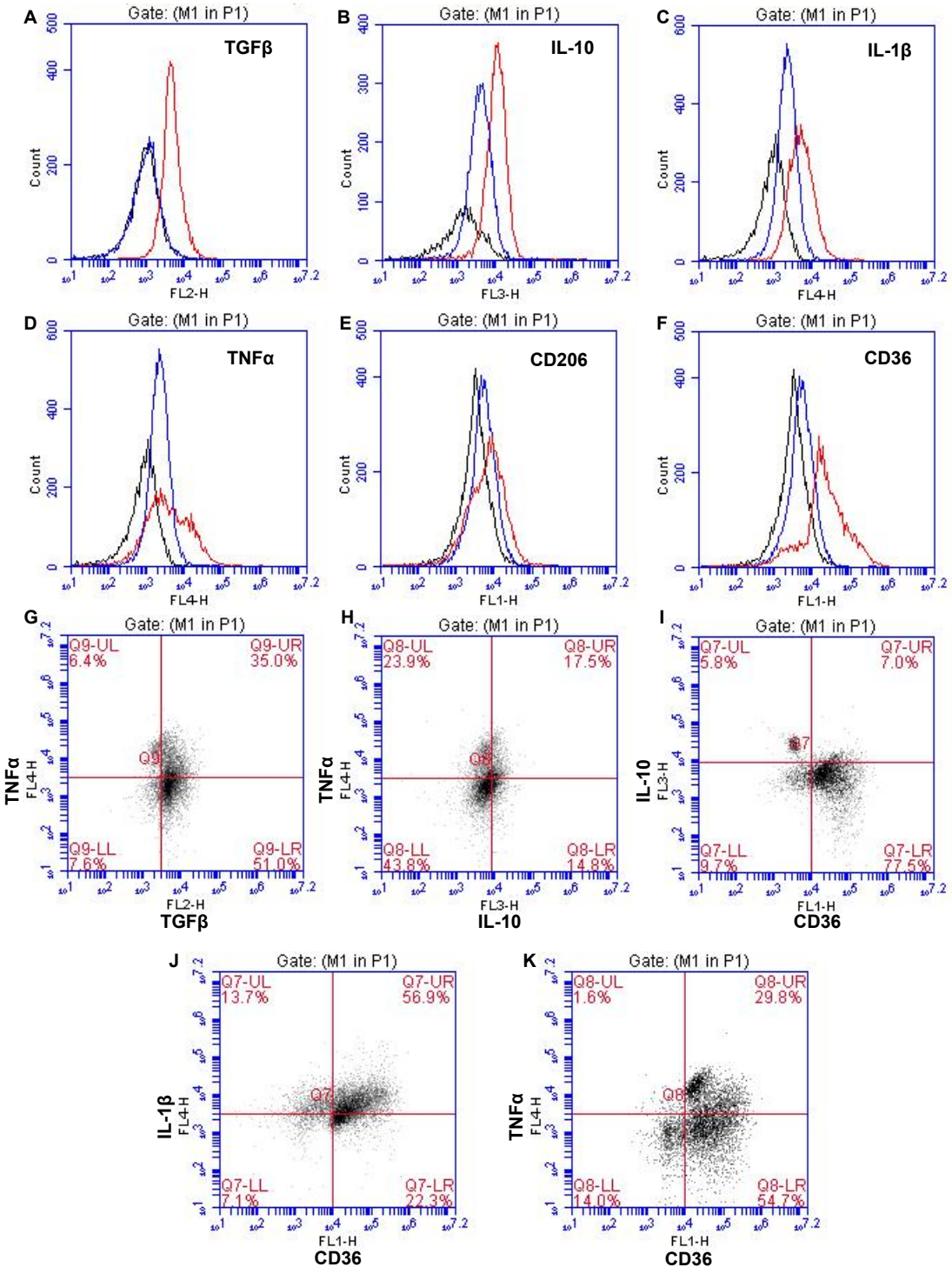


Figure 4. Muscle Mp phenotype. Mp were isolated from uninjured (0d) or lacerated gastrocnemius muscles at the indicated time points as described in Materials and Methods. As positive and negative controls, bone marrow derived Mp (right side of vertical bar in panels A-G) were activated with IFNγ and TNFα (M1), IL-4 (M2a), or IL-10 (M2c). Total RNA was isolated and reverse transcribed, and expression of IL-1β (A), TNFα (B), iNOS (C), CD206 (D), CD36 (E), TGFβ (F), and IL-10 (G) was analyzed by real-time PCR. M1-associated genes (A-C) are presented as expression relative to *in vitro* activated M1 Mp, and M2-associated genes (D-G) are presented as expression relative to *in vitro* activated M2a Mp. (H) Mp were isolated uninjured or injured muscles, equal numbers of Mp were incubated for 20 hours, and IL-10 secretion was measured by ELISA on the conditioned medium. Data did not pass tests of normality and equal variance, and are presented with center line as median, boxes representing the 25th and 75th percentiles, whiskers representing the 10th and 90th percentiles, and outliers as dots. * $p<0.05$ versus Mp from uninjured (0d) muscle. *In vitro* activated Mp were not included in statistical comparisons.

The expression of both M1 and M2a-associated genes observed in Mp from 3 day injured muscle may reflect the simultaneous presence of distinct M1 and M2a populations, or may be the result of a mixed Mp phenotype that is neither strictly M1 nor M2a. To distinguish between these possibilities, we analyzed intracellular cytokine content and cell surface activation markers by flow cytometry. At 3 days post-injury, muscle Mp contained intracellular TGFβ, IL-10, and IL-1β, and could not be separated into discrete high- and low-expressing populations based on these markers [Figure 5A-C]. TNFα exhibited a wider range of expression [Figure 5D]; however both TNFα-high and -low Mp exhibited similar levels of TGFβ and IL-10 expression [Figure 5G&H]. CD206 was barely detectable over non-specific isotype control levels [Figure 5E]. CD36 exhibited a wide range of expression [Figure 5F]; however, CD36-high and -low Mp exhibited similar levels of IL-10 and IL-1β expression [Figure 5I&J]. There was also no clear correlation, negative or otherwise, between expression of CD36 and expression of TNFα. While a distinct population of TNFα-high, CD36-mid Mp was observed, Mp at all levels of CD36 expression exhibited a wide range of TNFα expression [Figure 5K]. Taken together, these data indicated that muscle Mp at 3 days post-injury cannot be clearly divided into *in vitro*-defined M1 and M2a subpopulations. Instead, the mixed M1/M2a mRNA profile observed at 3 days post-injury appears to reflect a hybrid phenotype of individual muscle Mp.

Figure 5. Mp at 3 days after muscle laceration are not separable into M1 and M2a subsets. Cells were isolated from gastrocnemius muscles at 3 days post-injury and labeled for flow cytometry. Mp were gated as F4/80-positive cells. Histograms display Mp-specific expression of TGFβ (A), IL-10 (B), IL-1β (C), TNFα (D), CD206 (E), and CD36 (F). Red lines represent expression in a representative sample; black and blue lines represent control cells stained with F4/80 alone or with F4/80 and non-specific IgGs, respectively. Density plots display Mp expression of TNFα versus TGFβ (G), TNFα versus IL-10 (H), CD36 versus IL-10 (I), CD36 versus IL-1b (J) and CD36 versus TNFα (K). Cells in upper left quadrant in (I) are likely due to non-specific binding because this population was also seen in non-specific IgG-stained controls. Data are representative of 5 independent experiments of n=1-2 per experiment.


A
Gate: (M1 in P1)
TGFβ
Count
FL2-H
B
Gate: (M1 in P1)
IL-10
Count
FL3-H
C
Gate: (M1 in P1)
IL-1β
Count
FL4-H
D
Gate: (M1 in P1)
TNFα
Count
FL4-H
E
Gate: (M1 in P1)
CD206
Count
FL1-H
F
Gate: (M1 in P1)
CD36
Count
FL1-H
G
Gate: (M1 in P1)
Q9-UL 6.4%
Q9-UR 35.0%
Q9-LL 7.6%
Q9-LR 51.0%
TNFα
FL4-H
FL2-H
TGFβ
H
Gate: (M1 in P1)
Q8-UL 23.9%
Q8-UR 17.5%
Q8-LL 43.8%
Q8-LR 14.8%
TNFα
FL4-H
FL3-H
IL-10
I
Gate: (M1 in P1)
Q7-UL 5.8%
Q7-UR 7.0%
Q7-LL 9.7%
Q7-LR 77.5%
IL-10
FL3-H
FL1-H
CD36
J
Gate: (M1 in P1)
Q7-UL 13.7%
Q7-UR 56.9%
Q7-LL 7.1%
Q7-LR 22.3%
IL-1β
FL4-H
FL1-H
CD36
K
Gate: (M1 in P1)
Q8-UL 1.6%
Q8-UR 29.8%
Q8-LL 14.0%
Q8-LR 54.7%
TNFα
FL4-H
FL1-H
CD36

3. Cell therapy with M1 macrophages reduces muscle fibrosis after traumatic injury

While canonical M1 and M2a Mp do not appear to be present in lacerated muscle, *in vitro* studies have demonstrated that M1 Mp can enhance myoblast proliferation and decrease fibroblast collagen production (39,51). Therefore, we hypothesized that cell therapy with exogenous M1 Mp would improve muscle healing after laceration. Bone marrow-derived Mp were cultured and activated to an M1 phenotype, and were injected into lacerated gastrocnemius muscles at 7 days post-injury. In preliminary experiments, Mp were labeled with green fluorescent carboxyfluorescein succinimidyl ester (CFSE) prior to injection to determine how long the exogenous Mp remained in the muscle. Labeled Mp were easily detectable immediately and 1 day after injection [Figure 6]. By 3 days post-injection, labeled Mp were scarce and fluorescence was considerably reduced, and labeled Mp were undetectable by 5 days post-injection [Figure 6]. To ascertain that these results were not merely due to a loss of CFSE labeling in the injected Mp, tracking experiments were repeated using Mp from CD45.1 mice injected into muscles of wild-type (CD45.2) mice; flow cytometry analysis of CD45.1-positive cells similarly detected donor Mp in the injected muscles after 1 and 3 days, but not at 5 days (data not shown). These results demonstrate that injected Mp remain within the treated muscle for approximately 3 days.

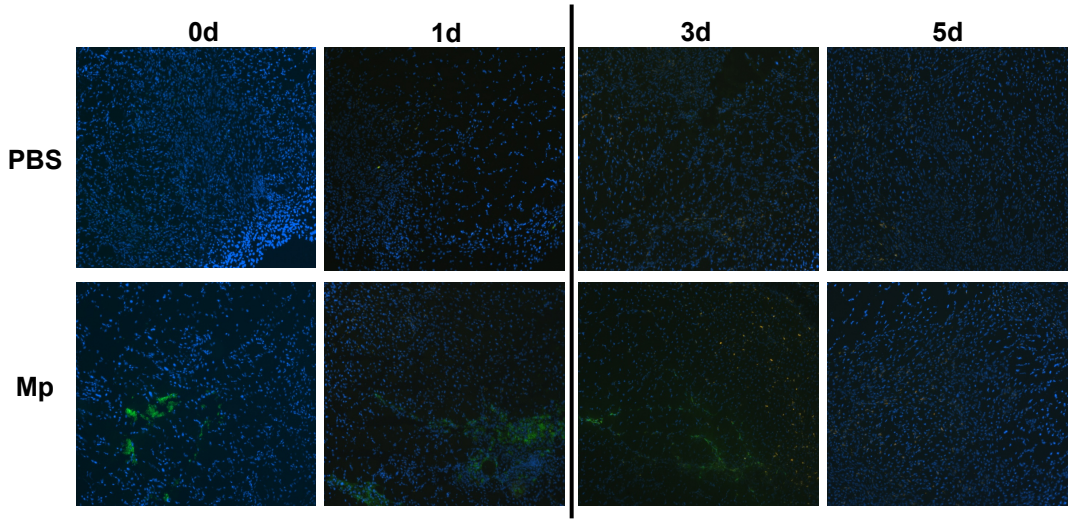


Figure 6. Injected Mp remain in the muscle for approximately 3 days. $0.5x10^6$ M1 Mp were labeled with CFSE, and labeled Mp or PBS vehicle was injected into lacerated gastrocnemius muscles at 7 days post-injury. Muscles were collected for histology at the indicated times after injection, and representative 20x images at each time point are shown. Images of muscles at 3d and 5d post-injection (right of vertical line) were taken with a longer exposure time than 0d and 1d images. CFSE label is shown in green, and nuclei are labeled with DAPI (blue).

Cell therapy with M1 Mp reduced collagen accumulation in lacerated muscles in a dose-dependent manner. Injection of $2x10^6$ M1 Mp reduced collagen accumulation at 14 days post-injury [Figure 7A&B]. Injection of $1x10^6$ M1 Mp produced a non-significant trend for reduced collagen accumulation, and 0.5 $x10^6$ M1 Mp had no effect [Figure 7B]. However, percent damaged area was not significantly reduced by any level of Mp cell therapy, and myofiber size and number were similarly unaffected [Figure 7C-E]. In contrast to the anti-fibrotic effects of M1 Mp cell therapy, injection of $2x10^6$ non-activated bone marrow-derived Mp did not alter collagen accumulation or regeneration [Figure 7F&G]. In

summary, cell therapy with exogenous M1 Mp produces a dose-dependent reduction in collagen accumulation in injured muscles but does not enhance regeneration.

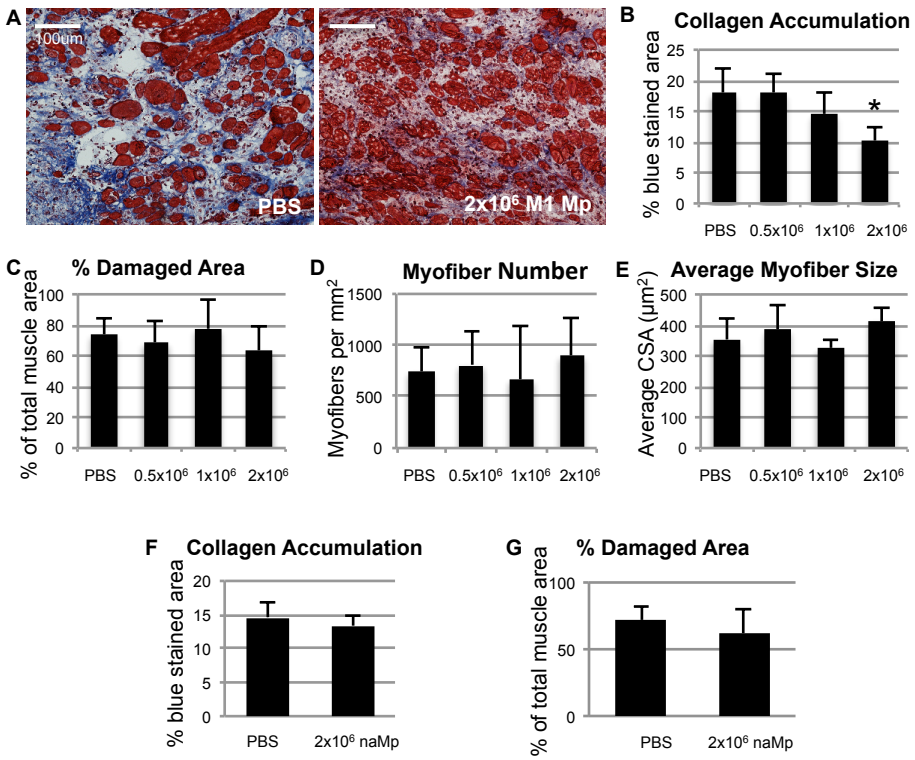


Figure 7. Cell therapy with M1 Mp reduces collagen accumulation but does not improve regeneration. (A-E) M1-activated bone marrow-derived Mp or PBS vehicle were injected into lacerated muscles at 7 days post-injury and analyzed at 14 days post-injury. (A) Representative trichrome images of lacerated muscles treated with PBS (left) or 2 x10^6 M1 Mp (right). (B) Collagen accumulation in muscles injected with PBS vehicle or with 0.5x10^6, 1x10^6, or 2x10^6 M1 Mp. (C) Damaged area as percent of total muscle area. (D) Average cross-sectional area of individual myofibers. (E) Number of myofibers per mm^2. (F&G) 2x10^6 non-activated (na) bone marrow-derived Mp or PBS vehicle were injected into lacerated gastrocnemius muscles at 7 days post-injury and analyzed at 14 days post-injury. (F) Collagen accumulation. (G) Damaged area as percent of total muscle area. Data are presented as mean +/- SD. * $p<0.05$ versus PBS control.

D. DISCUSSION

Mp are essential for efficient muscle regeneration but can also contribute to fibrosis across numerous tissues. These contrasting roles of Mp during tissue repair may be due to the ability of Mp to assume a variety of functional phenotypes upon exposure to different stimuli. Data from the present study demonstrate prolonged Mp accumulation during fibrotic healing after traumatic muscle injury. However, Mp in injured muscle exhibited neither an M1 nor an M2a phenotype. Instead, Mp at early time points after injury expressed a mix of both M1- and M2a associated markers, followed by a decrease in expression of most markers examined. In particular, IL-10 mRNA and protein secretion were highly elevated in Mp from 3 day injured muscle. Cell therapy with M1 Mp reduced collagen accumulation in injured muscles but had no significant effect on myofiber regeneration. These findings improve our understanding of Mp phenotypes during *in vivo* tissue repair, and provide insight into potential Mp-based therapies to improve muscle healing.

The supposition that M2a Mp perform tissue repair or wound healing functions *in vivo* has become increasingly accepted (7-9,88). In cell culture, M2a Mp produce a variety of factors that are important for tissue repair, including TGFβ, arginase, and numerous growth factors and ECM components (9,51,71). *In vivo*, M2a “markers” such as CD206 and arginase are expressed by Mp in skin wounds (42,64), dystrophic muscle lesions (62,76), and ischemic kidneys (96). However, while M2a Mp are defined in vitro by IL-4/-13 activation, neither IL-4 nor its receptor are required for expression of CD206 by skin wound Mp (64).

Additionally, CD206 is expressed at similar levels by Mp with both high and low expression of the M1-associated cytokine TNFα (64). These data indicate that *bona fide* M2a Mp are not present in murine skin wounds (74), and caution against extrapolating a complete *in vivo* Mp phenotype from a small number of phenotypic “markers” such as CD206.

The present study assessed a battery of mRNA and protein markers associated with M1 and M2a activation, and found that Mp after muscle laceration injury do not exhibit either strictly M1 or M2a phenotypes. Instead, both M1 and M2a markers are expressed simultaneously by Mp at 3 days post-injury, followed by a decrease in expression of most markers examined. At 3 days post-injury, muscle-derived Mp were comparable to cultured M1 Mp in expression levels of numerous genes, including IL-1β, TNFα, CD206, CD36, and TGFβ [Figure 4]. However, the very high expression of IL-10 and complete absence of iNOS in 3 day muscle Mp indicate that these early Mp are not entirely of the M1 phenotype. Additionally, contrary to our hypothesis of an M1-to-M2a switch, Mp expression of the M2a-associated genes CD36 and TGFβ decreased with time, while CD206 expression was not significantly elevated over resident Mp expression at any time point and was very low compared to cultured M2a Mp. In summary, data from the present study indicate that, during repair of skeletal muscle laceration, Mp transition from an early hybrid phenotype expressing both M1- and M2-associated markers to an apparently deactivated phenotype during the later stages of repair.

At 3 days post-injury, muscle Mp exhibited high levels of IL-10 mRNA expression and protein secretion, which may contribute to the apparent Mp deactivation observed at subsequent time points. IL-10 is a powerful anti-inflammatory cytokine that can suppress M1 activation of Mp (45,129,9) and may also inhibit some aspects of M2a activation (49). In addition to modulating Mp activation by other cytokines, IL-10 exposure *in vitro* produces an “M2c” phenotype (10); however, the mRNA expression profile of muscle Mp at 14 days post-injury bore only a partial resemblance to *in vitro* activated M2c Mp [Figure 3]. Specifically, while M2c Mp and 14 day muscle Mp both exhibited low expression of pro-inflammatory cytokines and CD36, expression of TGFβ and CD206 was considerably lower in 14 day muscle Mp versus M2c Mp. These data, while limited, suggest that muscle Mp during the later stages of repair are not analogous to *in vitro* activated M2c Mp, despite the ample production of IL-10 by muscle Mp during earlier stages of repair. Future studies will determine whether IL-10 production by muscle Mp contributes to their subsequent deactivation.

While the Mp populations present after muscle injury do not conform to M1/M2 classification, knowledge of the actual phenotypes present may provide insight into strategies for improving healing. For example, the apparent deactivated phenotype present during the later stages of repair suggests that increasing Mp activation may improve healing. This could be potentially be accomplished by cell therapy with *in vitro*-activated Mp. Indeed, Mp cell therapy has been useful in improving healing of numerous tissues, and activation of

these Mp to an M1, M2a, or other state may alter their ability to promote repair (108,110,113,115,116,130). In the present study, M1-activated bone marrow-derived Mp reduced collagen accumulation in injured muscle, while non-activated Mp had no effect. In contrast, in a mouse model of chronic renal inflammatory disease, transfusion of M1 Mp increased fibrosis and renal injury, while treatment with M2a Mp reduced fibrosis, and non-activated Mp had no effect (108). Prior Mp activation may not be necessary to promote repair in all tissues, however, as naive bone marrow-derived Mp reduced myofibroblast accumulation and fibrosis in a mouse model of liver injury (110). A variant of Mp cell therapy has also been used to enhance healing of human wounds. Hypoosmotically activated blood cell suspensions, which contain mainly monocyte/Mp and other leukocytes (114), enhance healing of ulcers in elderly and diabetic patients (113,115,130) and also reduce mortality and duration of hospitalization in patients with infected sternal wounds (116). Together, these studies in rodents and humans highlight the promise of Mp cell therapy for enhancement of tissue repair, but also indicate that the ideal Mp phenotype may differ based on the specific needs of the damaged tissue.

In conclusion, this study demonstrates that, contrary to the growing belief that M2a Mp are involved in *in vivo* tissue repair, Mp during repair of traumatic skeletal muscle injuries do not correspond to *in vitro*-defined M1 or M2a phenotypes. Instead, muscle Mp exhibit a transition from an early hybrid phenotype to a subsequent deactivated state, possibly due to Mp production of IL-10. Because the functions of such hybrid Mp are difficult to predict, we suggest

that, for future studies, investigation of Mp functions important for tissue repair, such as phagocytosis or matrix remodeling, may prove more useful than attempts to match endogenous tissue repair Mp to *in vitro*-defined phenotypic categories that may not actually exist *in vivo*. However, our finding that M1 Mp decreased fibrosis of lacerated muscle indicates that *in vitro*-activated Mp may prove useful as exogenous cell therapy. An improved understanding of the reciprocal regulation of Mp phenotype and the tissue repair environment may help to develop and refine Mp-based therapies to promote healing.

CHAPTER III

MACROPHAGES ALTER MYOBLAST AND FIBROBLAST ACTIVITY IN VITRO INDEPENDENTLY OF UROKINASE TYPE PLASMINOGEN ACTIVATOR SECRETION

A. INTRODUCTION

1. Macrophage modulation of myoblast activity

Numerous studies have demonstrated that macrophages (Mp) are essential for skeletal muscle regeneration (3,14,39). In addition to their classical function of phagocytosing dead tissues, Mp actively promote skeletal muscle regeneration by modulating myoblast activity. For instance, cell-cell contacts between Mp and myoblasts enhance survival of both cell types (16). Mp also produce soluble factors that enhance myoblast proliferation (16,17). Additionally, Mp of different phenotypes have different effects on myoblast activity: coculture with M1 Mp increases myoblast proliferation, while M2a Mp enhance differentiation and fusion (39). However, the mechanisms by which Mp alter myoblast activity have not been identified.

2. Macrophage modulation of fibroblast activity

While skeletal muscle regeneration is dependent on proliferation and differentiation of myoblasts, simultaneous activation of fibroblasts leads to formation of a collagenous scar at the injury site (20,23). In addition to their

effects on myoblasts, Mp are also capable of modulating fibroblast activity. Secreted factors produced by Mp can increase fibroblast proliferation *in vitro* (131,132). Additionally, in coculture, fibroblast collagen production is reduced by M1 Mp and increased by M2a Mp (51). TGFβ production by M2a Mp may be responsible for increasing collagen production (51). However, the mechanism by which M1 Mp reduce collagen production remains to be identified. In particular, it is not known whether M1 Mp produce as-yet-unidentified soluble factors that reduce fibroblast collagen expression, or whether cell-cell contacts are required.

3. Urokinase type plasminogen activator regulates myoblast and fibroblast activity

A potential molecular mediator produced by M1 Mp is the urokinase type plasminogen activator (uPA) (14,18). uPA increases myoblast proliferation through proteolytic activation of hepatocyte growth factor (HGF), a known myoblast mitogen (127). HGF also reduces collagen production in fibroblast cultures (133). uPA has been assigned both pro- and anti-fibrotic roles in different tissues (134,135), but appears to oppose fibrosis in skeletal muscle (13,15,136). In culture, endogenous uPA may decrease fibroblast collagen production (137). Thus, uPA is known to alter activity of both myoblasts and fibroblasts. We therefore hypothesized that Mp-derived uPA is responsible for the Mp-induced increase in myoblast proliferation and decrease in fibroblast collagen production observed *in vitro*.

B. MATERIALS AND METHODS

1. Animals.

Wild-type C57BL/6 mice were obtained from Harlan (Indianapolis, IN) and bred in our animal facility. Mice were housed at 22-24°C on a 12:12h light-dark cycle and provided with food and water ad libitum. Mice were used for bone marrow harvest at age 10-15 weeks. The Animal Care Committee at the University of Illinois at Chicago approved all experimental procedures.

2. Macrophage-conditioned medium

Bone marrow derived Mp were cultured as previously described (14). Briefly, bone marrow was flushed from mouse femurs and tibias using DMEM supplemented with 10% heat-inactivated FBS, 10% L929 cell-conditioned medium (source of M-CSF), 2mM L-glutamine, and 1% penicillin/streptomycin (Sigma-Aldrich). After 4 days, cells cultured in this manner are >95% F4/80+ by flow cytometry (14). On day 4, medium was changed to DMEM supplemented with 1% FBS, 2mM L-glutamine, and 1% penicillin/streptomycin, and cultures were either treated with 10ng/ml each IFNγ and TNFα (Peprotech, Rocky Hill, NJ) to obtain M1 Mp or not exposed to cytokines to obtain non-activated Mp. After 48h, conditioned medium (CM) was harvested from non-activated and M1 Mp and stored at -80°C.

3. Myoblast culture

C2C12 myoblasts (ATCC, Manassas, VA) were grown overnight on coverslips in DMEM supplemented with 1% FBS, 2mM L-glutamine, and 1% penicillin/streptomycin. Cultures were then switched for 24 hours to one of the eight experimental conditions summarized in Table II. These conditions included high serum positive control (Condition 1) and low serum negative control (Condition 2), and CM from M1 Mp and non-activated Mp (Conditions 3 & 4). Condition 5 was included to verify that any observed increase in myoblast proliferation with M1 Mp-CM was not due to residual activating cytokines. In a separate experiment, recombinant plasminogen activator inhibitor (PAI)-1 (Molecular Innovations, Novi, MI) was added to LS and LS+M1 Mp-CM conditions at concentrations of 0, 10, or 100 ng/mL. After 23 hours, 100μM BrdU was added for the final hour of culture to measure myoblast proliferation.

TABLE II. EXPERIMENTAL CONDITIONS FOR MYOBLAST CULTURES**.**

Experimental Condition #	**FBS**	**Mp-CM addition**	**IFNγ/TNFα**
1	10%	None	None
2	1%	None	None
3	1%	20% M1 Mp-CM	None
4	1%	20% non-activated Mp-CM	None
5	1%	None	2ng/mL each

4. Myoblast proliferation

BrdU incorporation was determined by immunofluorescence as previously described (26). Briefly, cells were fixed in cold acetone, washed in PBS, and incubated in 2 N HCl. Samples were neutralized with basic PBS (pH 8.5) and then washed with neutral PBS (pH 7.6). This was followed by incubation in 0.1% IGEPAL and then blocking buffer. Proliferating cells were labeled with a BrdU antibody for 1 h (1:10, Roche Diagnostics, Indianapolis, IN), then washed with PBS subsequently incubated with FITC anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA). Coverslips were then mounted in Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) to visualize nuclei. Myoblast proliferation was quantified as the percentage of BrdU-positive nuclei in three 20x fields per sample using an Eclipse 80i microscope with DS-Fi1 camera and NIS-Elements BR software (Nikon, Melville, NY).

5. Fibroblast culture and collagen expression

NIH-3T3 fibroblasts (ATCC) were grown overnight in DMEM supplemented with 10% FBS, 2mM L-glutamine, and 1% penicillin/streptomycin (high serum medium), then switched for 48 hours to high serum medium with or without 20% M1 Mp-CM. To determine collagen gene expression, total RNA was isolated from fibroblasts using the RNEasy Kit (Qiagen, Valencia, CA) following the manufacturer's instructions, equal amounts of RNA were reverse-transcribed using the Thermoscript RT-PCR system (Invitrogen), and PCR was performed for

Col1a1, Col1a2, and Col3a1. These isoforms were examined because collagens type I and type III contribute to fibrosis after skeletal muscle injury (138). Collagen gene expression was measured by densitometry and normalized to expression of GAPDH and to the untreated condition. See Table III for primer sequences.

TABLE III. PRIMER SEQUENCES FOR COLLAGEN GENE EXPRESSION.

Gene	**Forward Sequence, 5' to 3'**	**Reverse Sequence, 5' to 3'**
Col1a1	GAGTTTCCGTGCCTGGCCCC	TCTCACCGGGCAGACCTCGG
Col1a2	AGAACCCTGCTCGCACGTGC	CCTGGGCGCGGCTGTATGAG
Col3a1	CCTGGACGACCTGGAGAGCGT	GCCGTTATTCCCGGGAGGGC
GAPDH	TCTGACGTGCCGCCTGGAGA	GGGGTGGGTGGTCCAGGGTT

6. Statistical Analysis

Statistical analysis was performed using SigmaPlot version 12.0 (Systat Software, San Jose, CA). Data were analyzed by Student's t-test for two-group comparisons, or by one-way ANOVA and Student-Newman-Keuls post-hoc method for multiple comparisons. Data are presented as mean +/- standard deviation. $p<0.05$ was taken to indicate statistical significance.

C. RESULTS

1. M1 macrophage-conditioned medium increases myoblast proliferation.

Consistent with previous studies (39,139), CM from M1 Mp increased myoblast proliferation versus low serum control [Figure 8A]. Additionally, M1 activation was required for this effect, because CM from non-activated Mp did not increase myoblast proliferation. The M1 activator IFNγ is also known to increase myoblast proliferation (26,140). Therefore, to ascertain that a Mp-secreted factor, rather than residual activating IFNγ in the Mp medium, was responsible for the M1-induced proliferation, we also incubated myoblasts in low serum medium with 2ng/mL each IFNγ and TNFα, the maximum concentration of these cytokines that could have been carried over from the initial activation of the Mp culture (since Mp were activated with 10ng/mL, and 20% Mp-CM was added to myoblasts). We found that this concentration of IFNγ and TNFα did not significantly alter myoblast proliferation. Together, these data indicate that soluble factor(s) produced by M1 Mp promote myoblast proliferation, and M1 activation is necessary for this effect.

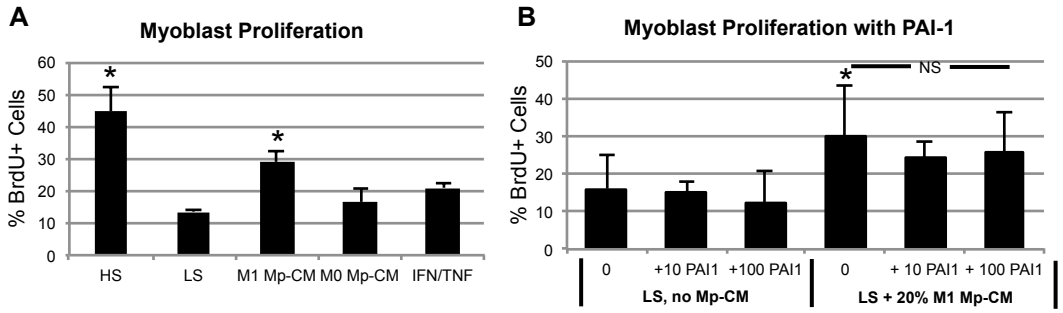


Figure 8. M1 Mp-CM increases myoblast proliferation independently of uPA. (A) C2C12 myoblasts were grown for 24 hours in 10% FBS (high serum, HS), 1% FBS (low serum, LS), LS supplemented with 20% CM from M1 or non-activated (M0) Mp, or LS supplemented with 2ng/mL IFNγ and TNFα. (B) C2C12 myoblasts were grown for 24 hours in LS or LS supplemented with 20% M1 Mp-CM, with PAI-1 added at 0, 10, or 100 ng/mL. BrdU was added for the last hour of culture, and myoblast proliferation was measured as percent of nuclei that were BrdU+. Data are presented as mean +/- SD. * $p<0.05$. NS = not significant.

2. M1 macrophage-conditioned medium reduces fibroblast collagen expression.

M1 Mp also reduce fibroblast collagen production in coculture experiments (51). However, these experiments did not determine whether cell-cell contact is required, or whether a secreted factor is responsible. We found that CM from M1 Mp reduced fibroblast expression of Col1a1 and Col3a1 mRNA, but did not alter expression of Col1a2 [Figure 9]. These results indicate that soluble factors produced by M1 Mp are sufficient to reduce fibroblast collagen expression.

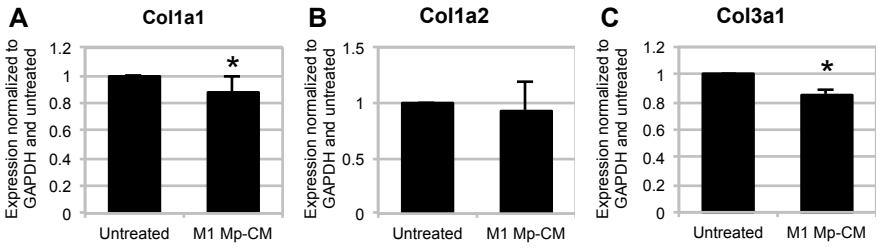


Figure 9. M1 Mp-CM reduces fibroblast collagen expression. 3T3 fibroblasts were cultured with or without 20% CM from M1 Mp. mRNA expression of Col1a1 (A), Col1a2 (B), and Col3a1 (C) were normalized to GAPDH expression of the same sample, then normalized to the untreated control condition. Data are presented as mean +/- SD. * $p<0.05$.

3. Secretion of urokinase type plasminogen activator is not required for M1 macrophage effects on myoblasts and fibroblasts.

Because uPA is able to modulate both myoblast and fibroblast activity (127,137), we hypothesized that uPA secretion by M1 Mp was responsible for the observed effects of M1 Mp-CM on these cells. However, despite previous studies that demonstrated increased Mp expression of uPA after M1 activation (14,18), uPA activity was below detectable levels in CM from M1 Mp (not shown). Additionally, the uPA inhibitor PAI-1 had no effect on myoblast proliferation in low serum conditions, either with or without M1 Mp-CM [Figure 8B]. These data indicate that soluble uPA is not necessary for M1 Mp to promote myoblast proliferation or reduce fibroblast collagen expression *in vitro*.

D. DISCUSSION

Mp are essential for skeletal muscle regeneration and for efficient repair of other tissues; however, Mp can also either promote or inhibit fibrosis, possibly dependent on the specific injury context and the mode of Mp activation (2,3,5,12,44). In cell culture, M1 Mp can both increase myoblast proliferation (16,17,39) and decrease fibroblast collagen production (51). We hypothesized that these effects are due to Mp secretion of uPA, which has pro-regenerative and anti-fibrotic properties after skeletal muscle injury *in vivo* (13,15,127). We found the M1 Mp-induced increase in myoblast proliferation and decrease in fibroblast collagen production are due to secreted factors. However, active uPA was not detected in M1 Mp CM, and PAI-1 had no effect on M1 Mp-CM-induced myoblast proliferation. Therefore, soluble uPA does not appear to be responsible for M1 Mp effects on myoblast and fibroblast activity *in vitro*.

Mp appear to be an important source of uPA during muscle regeneration in mice (127). However, in the present study, mouse bone marrow-derived M1 Mp did not produce detectable amounts of active soluble uPA. In a previous study from our laboratory, M1 activation of Mp increased mRNA expression of uPA (14); the only substantial differences between the methods used in the previous and present studies are that the previous study activated the Mp with LPS while the current study used IFNγ and TNFα, and the previous study measured mRNA expression while the present study measured uPA activity. Thus, the different results obtained could reflect differential activation of Mp by LPS versus IFNγ/TNFα. Additionally, uPA is subject to significant post-

translational regulation; uPA is secreted as a pro-enzyme that requires proteolytic cleavage for activation (141). Furthermore, the proteolytic activity of active uPA can be blocked by its endogenous inhibitor PAI-1 (141). These multiple levels of regulation may explain why M1 Mp-CM contains no detectable active uPA despite robust expression at the mRNA level.

Though cultured M1 Mp did not secrete active uPA in this study, these results do not preclude the possibility that Mp production of uPA *in vivo* promotes myoblast proliferation and muscle regeneration. Mp at early time points after traumatic muscle injury exhibit a hybrid phenotype that it not strictly M1 or M2a (data presented in this dissertation, and manuscript in preparation); therefore cultured M1 Mp and *in vivo* muscle repair Mp likely exhibit differences in their secretory profiles. While secretion of active uPA by endogenous muscle Mp has not been specifically examined, muscle Mp after injury do express uPA mRNA (127), and Mp-specific over-expression of uPA is sufficient to restore muscle regeneration in uPA-null mice, which otherwise exhibit severe defects in regeneration (13). Future studies may elaborate on the potential role of endogenous Mp-derived uPA in promoting myoblast proliferation and skeletal muscle regeneration *in vivo*.

A previous study demonstrated that coculture with M1 Mp reduces fibroblast collagen production (51), but did not determine whether soluble factors or direct cell contact were responsible. In the present study, we found that a soluble factor produced by M1 Mp inhibits fibroblast collagen expression. M1 Mp-CM has been demonstrated to enhance skeletal muscle regeneration in mice

(17), and the results of our present study suggest that this therapy may also prove useful in ameliorating fibrosis of skeletal muscle and other tissues.

CHAPTER IV

CONCLUSIONS AND DIRECTIONS FOR FUTURE RESEARCH

Mp are critical mediators of regeneration and fibrosis across numerous tissues. Functions of Mp during tissue repair include host defense, phagocytosis of necrotic tissue, secretion of growth factors and proteases, and promotion of angiogenesis and matrix remodeling (43,74,88). However, Mp can also contribute to scarring and fibrosis (12,43,44). These contrasting roles of Mp are likely related to the remarkable plasticity of these cells. Exposure to different cytokines, bacterial products, immune complexes, or other factors can produce markedly different patterns of gene expression and functional capacity (9). Because M2a activation increases Mp production of various factors known to be important for tissue repair, M2a Mp are frequently termed “wound healing” Mp, and are widely assumed to participate in tissue repair *in vivo* (7-9,88). However, the actual *in vivo* phenotypes of Mp during tissue repair have only recently begun to be characterized.

Data presented in this dissertation indicate that Mp after traumatic muscle injury cannot be categorized as strictly M1 or M2a. Instead, muscle Mp after injury exhibit an early hybrid phenotype expressing both M1 and M2a markers, followed by a general deactivation. These data contradict the notion that M2a Mp are present during skeletal muscle repair, or that Mp exhibit an M1 to M2a transition. Furthermore, these data indicate that the presence of a single M1 or M2a marker on Mp *in vivo* is insufficient to extrapolate a complete Mp phenotype.

For example, Mp expression of iNOS or CD206, respectively, has often been assumed to indicate an M1 or M2a phenotype *in vivo* (76,96,98,126). In the present study, if one were to extrapolate muscle Mp phenotype from these two markers alone, one would erroneously conclude that muscle repair Mp are of an M2a phenotype, because CD206 is expressed and iNOS is not. However, based on expression of other phenotypic markers and the fact that CD206 is expressed only at low levels, muscle repair Mp are clearly not of a strictly M2a phenotype at any time point. In general, expression of one or a few phenotypic markers *in vivo* does not necessarily indicate that the Mp was activated by the cytokine(s) which upregulate that marker *in vitro*, or that other aspects of the predicted phenotype will be present.

Previous studies have found that the canonical M1/2a/b/c phenotypes are by no means the only phenotypes that Mp can assume, both *in vitro* and *in vivo*. Upon exposure to combinations of different activating cytokines, even cultured Mp exhibit complex phenotypes that are distinct from those produced by exposure to either cytokine alone (45). Additionally, Mp exhibit significant temporal plasticity; even a single activating cytokine can produce different gene expression patterns at different durations of exposure, and Mp remain responsive to additional stimuli after their initial activation (45,58). During tissue repair *in vivo*, Mp are exposed to a plethora of potential activating stimuli, which likely change throughout the repair process; therefore it should not be surprising to find that Mp phenotypes *in vivo* do not strictly correspond to *in vitro* phenotypes. In a mouse skin wound model, markers associated with both M1 and M2a activation

were observed at both early and late stages of repair, frequently on the same cell simultaneously (64). Additionally, the M2a activation markers CD206, Ym1, and dectin-1 exhibited entirely different temporal expression patterns, and expression of these M2a-associated markers was not dependent on the canonical M2a activator IL-4 and its receptor IL-4Rα (64). The authors concluded that wound Mp exhibit temporally regulated and heterogeneous phenotypes expressing both M1 and M2a-associated markers. Data presented in this dissertation support this conclusion, though the expression patterns of specific cytokines and surface markers appear to differ between Mp from lacerated muscle versus the skin wound model.

Differences of Mp phenotype during healing of skeletal muscle laceration versus skin wounding may be due to the different microenvironments to which Mp are exposed in these two tissues. However, the specific factors involved in activation and modulation of Mp phenotype during tissue repair *in vivo* remain to be determined. IFNγ promotes certain characteristics of an M1-like phenotype in toxin-injured or dystrophic skeletal muscle (26,92). IFNγ also enhances regeneration and reduces fibrosis of lacerated skeletal muscle, though the effects on muscle Mp phenotype were not investigated (63). Other potential modulators of Mp phenotype after injury include necrotic tissues, reactive oxygen species, ECM fragments, and apoptotic cells. Phagocytosis of apoptotic cells reduces Mp expression of pro-inflammatory cytokines and increases expression of TGFβ *in vitro*, and similar anti-inflammatory effects appear to occur *in vivo* (142). Our present data demonstrate that another powerful anti-inflammatory mediator, the

cytokine IL-10, is highly expressed by Mp at 3 days after laceration injury. Future studies may determine whether IL-10 production is responsible for the apparent Mp deactivation observed at later time points after muscle laceration, and whether enhancing activation of endogenous Mp can improve muscle healing.

Because Mp activation appears to be generally suppressed during the later stages of muscle laceration repair, and because M1 Mp can promote myoblast proliferation and reduce fibroblast collagen production *in vitro* (39), we tested whether cell therapy with M1 Mp could improve muscle healing after laceration. Treatment with M1 Mp reduced collagen accumulation in lacerated muscle, but did not alter the number or size of regenerating myofibers. Mp injection was performed at 7 days post-injury; at this time point, regenerating myofibers are beginning to form at the injured site, but significant collagen accumulation has not yet occurred. While this study did not specifically examine myoblast proliferation *in vivo*, the increase in the number of regenerating myofibers between 7 and 14 days in untreated lacerated muscle indicates that significant proliferation is likely occurring during this time. It is possible that any mitogenic stimulus provided by exogenous M1 Mp was small compared to endogenous proliferative signals, and therefore did not produce a significant increase in myofiber number beyond that which occurs in the absence of therapy. Alternatively, it is possible that the *in vitro* mitogenic effects of M1 Mp do not translate to the *in vivo* situation, whether due to possible inhibitory factors present in the injured muscle, or to a change in phenotype of the exogenous Mp upon exposure to a new environment.

Despite the lack of effect of M1 Mp on myofiber regeneration, this therapy significantly reduced collagen accumulation in lacerated muscle. M1 Mp also reduce fibroblast collagen production in cocultures (51), though the molecular mechanisms underlying these effects remain to be identified. M1 Mp express a number of proteases that can degrade ECM, including MMP-7 and uPA (14,18). Because uPA can promote muscle regeneration and reduce fibrosis (13-15), we hypothesized that production of uPA by M1 Mp was responsible for the increase in myoblast proliferation and decrease in collagen production induced by Mp conditioned medium (CM). However, we found no detectable uPA activity in CM from M1 Mp, and PAI-1 did not reduce the effects of M1 Mp-CM on myoblast proliferation. Thus, the secreted factors produced by M1 Mp to modulate myoblast and fibroblast activity remain to be determined. Future studies may also determine the mechanism by which M1 Mp reduce collagen accumulation in lacerated skeletal muscle *in vivo*.

In summary, data presented in this dissertation demonstrate that endogenous Mp during repair of lacerated skeletal muscle exhibit temporally regulated phenotypes that cannot be classified as strictly M1 or M2a. Muscle Mp transition from an early hybrid phenotype expressing both M1- and M2a-associated markers to an apparently deactivated phenotype as repair progresses. Additionally, cell therapy with M1 Mp reduces fibrosis in lacerated muscle.

These findings are significant because they challenge an increasingly accepted but largely unsubstantiated opinion that M2a Mp are “wound healing”

Mp and contribute to tissue repair *in vivo*. This opinion is largely derived from cell culture studies, in which M2a Mp can produce factors important for tissue repair. However, *in vivo* studies often infer a complete M1 or M2a phenotype from the presence of just one or two phenotypic markers. The validity of this method is called into question by data presented in this dissertation, which demonstrate that Mp in injured muscle can adopt a hybrid phenotype with characteristics of both M1 and M2a activation. These findings indicate that extrapolation of Mp phenotype based only on the presence of a few markers provides at best an incomplete picture of *in vivo* Mp activation, and may even lead to erroneous conclusions. Despite the apparent absence of bona fide M1 and M2a Mp during skeletal muscle repair, these *in vitro*-defined phenotypes remain powerful tools for understanding and improving tissue repair. Data presented here demonstrate that treatment with exogenous M1 Mp reduces fibrosis in injured muscle; studies in other tissues have shown similar potential for Mp-based therapies to enhance repair, though it appears that the ideal repair-promoting Mp phenotype may differ among various tissues and pathologies. Additionally, tracking the phenotypic and functional changes of donor Mp upon introduction to tissue repair environments may provide insight into the mechanisms by which Mp activation is regulated *in vivo*. While myriad potential activating factors have been identified *in vitro*, the actual mechanisms of Mp activation and modulation during tissue repair remain to be elucidated. An improved understanding of the reciprocal regulation of Mp phenotype and the tissue repair environment may assist in development of novel therapies to promote healing.

CHAPTER V

APPENDIX

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committees (MC 672)
Office of the Vice Cancecllor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

January 11, 2010

Timothy J. Koh
Kinesiology and Nutrition
M/C 994

Dear Dr. Koh:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on **12/15/2009**. *The protocol was not initiated until final clarifications were reviewed and approved on* **1/8/2010**. *The protocol is approved for a period of* ***3*** years with annual continuation.

Title of Application: Macrohage Phenotype and Tissue Repair

ACC Number: 09-227

Initial Approval Period: 1/8/2010 to 12/15/2010

Current Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 1

Funding Agency	**Grant Title**			**Portion of Grant Matched**
AHA- American Heart Association	*Statin: Myopathy: Anti-Inflammatory Effects of Statins in Skeletal Muscle Repair*			*Matched*
Grant Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Grant PI**
0910011G	*Funded*	*2008-06806*	*UIC*	*Margaret Novak*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Richard D. Minshall, PhD
Chair, Animal Care Committee
RDM/*ss*
cc: BRL, ACC File, Margaret Novak, Rita Mirza, **PAF #2008-06806**

Phone (312) 996-1972 • Fax (312) 996-9088

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committee (OACIB) (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

12/15/2011

Timothy J. Koh
Kinesiology and Nutrition
M/C 994

Dear Dr. Koh:

The protocol indicated below was reviewed in accordance with the Animal Care Policies and Procedures of the University of Illinois at Chicago and renewed on 12/15/2011.

Title of Application: Macrophage Phenotype and Tissue Repair
ACC NO: 09-227
Original Protocol Approval: 1/8/2010 (3 year approval with annual continuation required).
Current Approval Period: 12/15/2011 to 12/15/2012

Funding: *Portions of this protocol are supported by the funding sources indicated in the table below.*
Number of funding sources: 3

Funding Agency	Grant Title			Portion of Grant Matched
AHA- American Heart Association	*Statin: Myopathy: Anti-Inflammatory Effects of Statins in Skeletal Muscle Repair*			*Matched*
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
09-10011G	*Funded*	*2008-06806*	*UIC*	*Margaret Novak*
Funding Agency	Grant Title			Portion of Grant Matched
American College of Sports Med.	*Activated Macrophages and Repair of Skeletal Muscle Injuries*			*Matched*
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
	Funded	*2011-03604*	*UIC*	*Margaret Novak*
Funding Agency	Grant Title			Portion of Grant Matched
NIH	*Macrophage Phenotype And Impaired Wound Healing*			*Matched*
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
RO1GM09-2850	*Pending*	*2011-02218*	*UIC*	*Timothy J. Koh*

Phone (312) 996-1972 • Fax (312) 996-9088

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.

Thank you for complying with the Animal Care Policies and Procedures of the UIC.

Sincerely,

Richard D. Minshall

Richard D. Minshall, PhD
Chair, Animal Care Committee

RDM/*kg*
cc: BRL, ACC File, Margaret Novak, Rita Mirza

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committee (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

November 11, 2011

Timothy J. Koh
Kinesiology and Nutrition
M/C 994

Dear Dr. Koh:

The modifications requested in modification indicated below pertaining to your approved protocol indicated below have been reviewed and approved in accordance with the Animal Care Policies of the University of Illinois at Chicago on *11/09/2011.*

Title of Application: Macrophage Phenotype and Tissue Repair

ACC Number: 09-227

Modification Number: 11

Nature of Modification: *PI is requesting 96 additional mice of new strain (B6.SJL-Ptprca Pepcb/BoyJ) to generate sufficient bone-marrow-derived macrophages for the treatment protocol as approved previously.*

Protocol Approved: *1/8/2010*

Current Approval Period: *12/15/10 to12/15/11. Protocol is eligible for 1 additional year of renewal prior to expiration and resubmission.*

Current Funding: *Portions of this protocol are supported by the funding sources indicated in the table below.*
Number of funding sources: 3

Funding Agency	Grant Title			Portion of Grant Matched
AHA- American Heart Association	*Statin: Myopathy: Anti-Inflammatory Effects of Statins in Skeletal Muscle Repair*			*Matched*
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
0910011G	*Funded*	*2008-06806*	*UIC*	*Margaret Novak*
Funding Agency	Grant Title			Portion of Grant Matched
American College of Sports Medicine	*Activated Macrophages and Repair of Skeletal Muscle Injuries*			*Matched*
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI

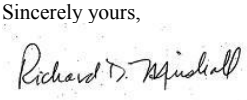
Phone (312) 996-1972 • Fax (312) 996-9088

	Funded	*2011-03604*	*UIC*	*Margaret Novak*
Funding Agency	Grant Title			Portion of Grant Matched
NIH	*Macrophage Phenotype And Impaired Wound Healing*			*Match 3*
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
RO1GM092850	*Pending*	*2011-02218*	*UIC*	*Timothy J. Koh*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.

Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,



Richard D. Minshall, PhD
Chair, Animal Care Committee

RDM/*ss*
cc: BRL, ACC File, Margaret Novak, Rita Mirza

CHAPTER VI

CURRICULUM VITAE

Margaret Louise Novak

EDUCATION

2012 Ph.D., Movement Sciences,
University of Illinois at Chicago

2002 B.S., Mathematics, Specialization in Computer Science
The University of Chicago

RESEARCH, TEACHING, AND PROFESSIONAL EXPERIENCE

2006-2011 Research & Teaching Assistant,
Department of Kinesiology and Nutrition
University of Illinois at Chicago

2002-2006 Personal Trainer, NSCA Certified
Bally Total Fitness, Chicago IL

2000-2002 Teaching Assistant, Department of Mathematics
The University of Chicago

HONORS AND AWARDS

2011-2012 Dean's Scholar Award, University of Illinois at Chicago

2009 Young Investigator Award, Gordon Research Conference on Tissue Repair and Regeneration

2002 Phi Beta Kappa, The University of Chicago

GRANTS

Activated Macrophages and Repair of Skeletal Muscle Injuries. (2011-2012).

Role: PI. Doctoral student research grant funded by the American College of Sports Medicine. The primary aim of this project is to investigate the role of classically and alternatively activated macrophages in skeletal muscle regeneration and fibrosis, and to use macrophage cell therapy to improve regeneration.

Statin Myopathy: Anti-Inflammatory Effects of Statins in Skeletal Muscle Repair. (2009-2010). Role: PI. Pre-doctoral Fellowship funded by the American Heart Association. The primary aim of this project is to determine whether simvastatin impairs skeletal muscle regeneration through anti-inflammatory effects on macrophages.

PUBLICATIONS

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INVITED TALKS

Simvastatin delays muscle regeneration in mice. Presented at the Gordon Research Conference on Tissue Repair and Regeneration, New London, NH, June, 2009.

POSTER PRESENTATIONS

Macrophage-specific expression of uPA promotes skeletal muscle regeneration. Presented at Gordon Research Conference on Tissue Repair and Regeneration, New London, NH, June 2011.

uPA and macrophages in skeletal muscle regeneration. Presented at Experimental Biology 2010, Anaheim, CA, April 2010.

Simvastatin delays muscle regeneration in mice. Presented at the Gordon Research Conference on Tissue Repair and Regeneration, New London, NH, June 2009.

COX-2 inhibitor reduces skeletal muscle hypertrophy in mice. Presented at the Adult Skeletal Muscle Symposium, Lexingon, KY, October 2008.

SYMPOSIA

Engineering better repair/regeneration: An eye towards therapeutics and the clinic II, Discussion Leader, Gordon Research Seminar on Tissue Repair and Regeneration, June 2011.

CHAPTER VII

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